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Metabolic analysis of four phenolic acids in rat by liquid chromatography–tandem mass spectrometry

Zi-chuan Zhang, Man Xu, Shi-feng Sun, Xue Qiao, Bao-rong Wang, Jian Han, De-an Guo*

The State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100083, China

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ABSTRACT

A liquid chromatography-diode array detection-electrospray ionization ion trap mass spectrometry (LC–DAD–ESI-MS^n) method was established for the analysis of danshensu, caffeic acid, ferulic acid and isoferulic acid in rat plasma, bile, urine and feces after oral administration or intravenous injection. Liquid–liquid extraction was employed for the preparation of biosamples, and the chromatographic separation was carried out using an Agilent Zorbax Extend C_{18} reversed phase column and acetonitrile-0.1% formic acid as the mobile phase. Totally nineteen metabolites were detected and identified as prototype, methylated, hydroxylated, sulfated and glucuronized conjugates. The metabolism of the individual phenolic acids in biosamples was investigated, and the metabolic pathway was proposed. By comparing the metabolism of different compounds which shared similar structures, we were able to find that methylation was the main pathway of danshensu metabolism, and the double bond on the side chain was critical for the drug excretion via bile and the formation of glucuronized conjugates. The results proved that the established method was simple, sensitive and reliable, which could be used to detect and identify the structures of metabolites and to better understand their *in vivo* metabolism.

1. Introduction

Danshen, the dried roots of *Salvia miltiorrhiza*, is a widely used traditional Chinese medicine for the treatment of coronary heart disease, cerebrovascular disease, bone loss, hepatocirrhosis and chronic renal failure [1]. Phenolic acids, the water soluble constituents in Danshen, are thought to be the major active constituents because aqueous decoction is traditional used in China. Phenolic acids in Danshen could be divided into two groups: monomer and polymer [2]. Danshensu (DSS), caffeic acid (CA), ferulic acid (FA) and isoferulic acid (IFA) are the monomers in Danshen (see Fig. 1), and it has been reported that these monomers, especially DSS, could protect the cardiac muscle by reducing oxygen free radical generation, inhibiting peroxidative damage, preventing platelet aggregation and calcium antagonizing [3–7]. Therefore it is necessary to clarify the absorption, distribution, metabolism and excretion (ADME) of these monomers, which construct the polymers in Danshen.

Great interests have been focused on these monomers in Danshen because of their therapeutic potential. However, the metabolism of these compounds has not been well clarified. Although LC–MS had been widely used for the metabolism studies and structure analysis as a sensitive and reliable approach [8–10], most of the research had focused on the metabolism of certain constituents after dosing of Danshen crude drug or compound Danshen recipe. We have earlier reported the metabolic study on the total phenolic acids of Danshen in rat urine and feces, and detected and identified five metabolites [11]. Pei et al. explored the metabolism of DSS from Compound Danshen Dripping Pills in human serum, and found two acetylated metabolites [12]. Wang et al. and Wei et al. investigated the metabolism after dosing of Compound Danshen preparations [13,14]. However, metabolic research after dosing of compound recipe or crude herbal drugs could not explain the exact metabolic pathway of certain metabolites, and the previous study is quite incomplete to form a comprehensive understanding towards the metabolism of monomers in Danshen. To our knowledge, there has been no systematical report on the metabolism of these monomers in biosamples like rat plasma, urine, feces and bile.

In this paper, we report the identification of the metabolites of DSS, CA, FA and IFA in the plasma, urine, feces and bile after both oral administration and intravenous injection to rats. A liquid chromatography–tandem mass spectrometry method (LC–MS) was

* Corresponding author at: School of Pharmaceutical Sciences, Peking University Health Science Center, No. 38 Xueyuan Road, Beijing 100083, People's Republic of China. Tel.: +86 10 82802024; fax: +86 10 82802700.
E-mail address: gda@bjmu.edu.cn (D.-A. Guo).

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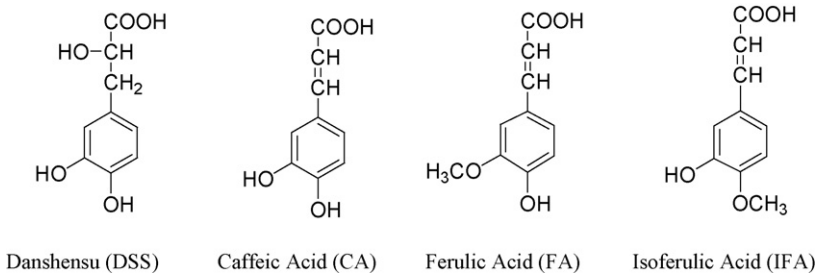

Fig. 1. The structures of DSS, CA, FA, and IFA.

adopted to elucidate the structure of the metabolites, and the difference among biosamples via different administration pathways was compared. This work was conducted by comparing the metabolites after dosing of single compounds, and the results provided a solid basis for the clarification of the metabolism of Danshen recipe.

2. Experimental

2.1. Materials and reagents

DSS was purchased from Sikehua Biotech Co. Ltd. (Sichuan, China) in the form of sodium danshensu. CA, FA and IFA were purchased from Yancheng Langde Chemical & Pharmaceutical Co. Ltd (Yancheng, China). The purities of the chemicals are not less than 98%.

HPLC-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA), and HPLC-grade methanol was from Tianjin Special Chemical Reagents Co. Ltd. (Tianjin, China). Ethyl acetate and formic acid were of analytical grade from Beijing Chemical Factory. Physiological saline was purchased from Beijing Double-Crane Pharmaceutical Co. Ltd. High-purity nitrogen (99.999%) and helium (99.999%) were obtained from the Gas Supplies Center of Peking University Health Science Center (Beijing, China).

2.2. Animals and drug administration

Male Sprague–Dawley rats (180–220 g) were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, China). Animals were kept in an environmentally controlled breeding room for 3 days and were fed with food and water *ad libitum*. Before the experiment, all the rats were fasted overnight but with free access to water. DSS, CA, FA and IFA were dissolved in physiological saline, respectively. The solution at 5 mg/ml was administrated orally at a dose of 40 mg/kg to rat, while the solution at 3 mg/ml was injected via tail vein at a dose of 6 mg/ml. Physiological saline was administered orally to the rats at a dose of 10 ml/kg body, or injected to the rat through the tail vein at 2 ml/kg for blank samples. Protocols of animal experiments had been approved by the Animal Center of Peking University Health Science Center.

2.3. Sample collection

Blood samples were collected by decapitation at 3 min after intravenous injection or 10 min after oral administration, respectively. For each compound four rats were divided into two groups for tail vein injection and oral administration. Plasma was obtained after centrifugation and was then stored at −80 °C before analysis.

For bile samples, four rats for each compound were fixed on wooden plate, and they were divided into two groups for oral administration or intravenous injection. Before the experiment, rats were anesthetized using pentobarbital sodium. Bile was conducted using bile duct cannulated with PE-10 tubing (ID is 0.8 mm, Becton Dickinson, U.S.A.) after incising the abdomen, which was then covered with saturated cotton. Drugs, or physiological saline for blank samples, were oral administrated or injected via tail vein when the rats were conscious, and bile was collected during 0–12 h. All bile samples were collected and stored at −80 °C before analysis.

Urine and feces samples were collected using metabolic cages. Two rats were put into one metabolic cage with free access to de-ionized water for each compound through each administration pathway. Urine and feces samples were collected over 0–12 h after drug administration. After urine samples were centrifuged, urine and feces were stored at −80 °C before analysis.

2.4. Sample preparation

Samples of plasma (2 ml), urine (2 ml) and bile (2 ml) were mixed with 10% (v/v) hydrochloric acid to reach the pH 2.0 in clean tubes, respectively. After vortex-mixing for 2 min, 6 ml of ethyl acetate was added to each tube, and extraction was performed by vortex-mixing for 5 min. After centrifugation at 9000 rpm for 5 min, 4.8 ml of ethyl acetate was transferred to a clean test tube. The residue was resolved in 4.8 ml of ethyl acetate and extracted again through the same method. Then the two supernatants were combined and dried under a flow of nitrogen gas at 35 °C. The residue was reconstituted in 300 μl of methanol, and filtered through a membrane (0.45 μm pore size). A 10 μl aliquot was injected into the LC–MS^n system for analysis.

Feces samples were crushed and weighed, and then mixed with methanol at the rate of 6 ml/g. Ultrasonic extraction was performed and the samples were then centrifuged at 9000 rpm for 5 min. Supernatants were transferred to clean tubes and dried under a flow of nitrogen gas at 35 °C. The residue was reconstituted in 300 μl of methanol, and filtered through a membrane (0.45 μm pore size). A 10 μl aliquot was injected into the LC–MS^n system for analysis.

2.5. Instrumentation and conditions

The HPLC analysis was carried out using an Agilent 1100 HPLC system with diode-array detector. The column configuration was composed of an Agilent Zorbax Extend C_{18} reversed phase column (5 μm, 250 mm × 4.6 mm) and an Agilent Zorbax Extend C_{18} guard column (5 μm, 12.5 mm × 4.6 mm). The mobile phase was gradient elution which was mixed with acetonitrile (A) and 0.1% (v/v) aqueous formic acid (B). The initial condition was A–B (5:95, v/v), and linearly changed to A–B (33:67, v/v) at 40 min. The column temperature was maintained at 20 °C, the flow rate was 0.8 ml/min, and detection wavelength was set at 288 nm.

The HPLC–MS^n detection was performed with an Agilent 1100 Series HPLC and Finnigan LCQ Advantage ion trap mass spectrometry equipped with an electrospray ionization source. The post-column splitting ratio of LC effluent which was introduced

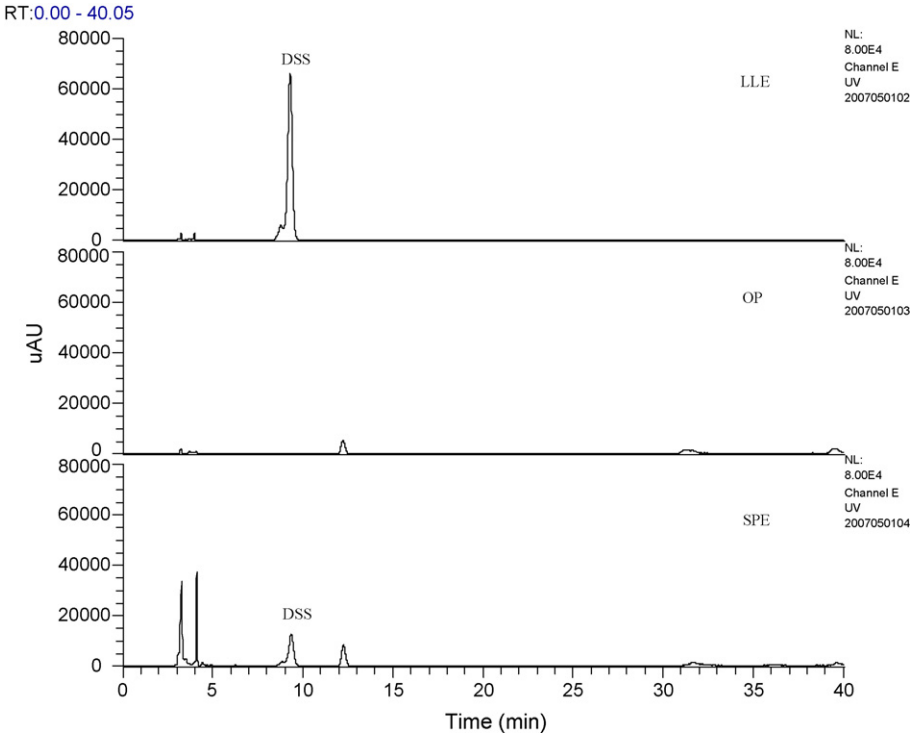

Fig. 2. The chromatograms of different extraction methods in rat plasma. Blank plasma sample spiked with danshensu standard after LLE: liquid–liquid extraction; OP: organic precipitation; SPE: solid-phase extraction.

into the ESI source was 2:1. High-purity nitrogen (N_2) was used as the nebulizer gas and ultra-high-purity helium (He) was the collision gas. The mass spectra were recorded in negative mode with the following parameters: sheath gas, 40 arbitrary units; auxiliary gas, 10 units; spray voltage, 4.5 kV; capillary temperature, 350 °C; capillary voltage, −10 V; tube lens offset, −10 V. The spectra were recorded from *m*/*z* 50–1000 for full-scan MS analysis. In order to select the two most abundant ions in turn in each MS scan, data-dependent acquisition was utilized. To acquire satisfactory product ion spectra, collision energy for CID was adjusted at 45% (units specific to ThermoFinnigan system), and the isolation width of precursor ion was set as 3.0 Th.

3. Results and discussion

3.1. Extraction, chromatography and mass spectrometry method

Different methods were tested for sample preparation, including liquid–liquid extraction (LLE) a solid-phase extraction (SPE) and organic precipitation (OP). Before the extraction was performed, we added standard DSS to blank biosamples and analyzed the samples with HPLC. Fig. 2 showed the extraction efficiency of different methods in rat plasma. In our test, SPE with Waters Oasis HLB cartridges and organic precipitation with methanol could not provide us with satisfactory recovery. Pan et al. reported that the recovery of phenolic acids through SPE was low because of the loss during washing [1], and Li et al. fully validated a LLE method coupled with liquid chromatography/tandem mass spectrometry for the analysis of six phenolic constituents of Danshen in human serum [15]. So LLE was utilized during sample extraction. Different extraction solvents, pH condition and extraction time were examined, and finally 10% hydrochloric acid was added to reach the pH 2.0 and the extraction was conducted twice with ethyl acetate in order to achieve better recovery and stability. Considering the unstable property of fecal constituents, we chose ultrasonic extraction and methanol was finally selected according to our previous work [9,16].

In the previous work, we found that MeCN–H_2O gave better separation than MeOH–H_2O for phenolic acids [10], so a MeCN–H_2O system was utilized as the mobile phase. Meanwhile, 0.1% HCO_2H was added to the mobile phase because acid could reduce the ionization of phenol, phenolic hydroxyl and carboxyl groups [16,17]. Other parameters were investigated, including column, column temperature, gradient, flow rate and detection wavelength. Symmetric peaks and better separation could be achieved at 20 °C with the flow rate of 0.8 ml/min and the detection wavelength of 288 nm using Zorbax Extend C_{18} column. Gradient elution changed linearly from acetonitrile-0.1% aqueous formic acid (5:95, v/v) to (33:67, v/v) at 40 min.

A LC–MS method was established for the metabolic study of phenolic acids. Standard DSS, CA, FA and IFA were analyzed for maximum intensity to get optimized instrument parameters of mass spectrometry, and the results were also used for the identification of the mother compounds and their metabolites in biosamples. To achieve satisfactory MS/MS fragmentation information for all the constituents in rat biosamples, the data-dependent scan was utilized and the two most abundant ions in each scan were selected and subjected to MS/MS analysis. The relative collision energy for CID was set at 45%.

3.2. Analysis of metabolites in rat by LC–MS^n

Blank plasma, bile, urine and feces were collected and compared with those after drug administration. In order to explain the difference between blank and biosamples after dosing of drugs, HPLC–MS^n technique was utilized to identify the possible structures of the metabolites. Under the established conditions, totally 19 compounds were detected after dosing of DSS, CA, FA and IFA, respectively, in rat plasma, urine and bile samples, while none was

Table 1
HPLC–MS^2 data and *in vivo* identification of metabolites in rat plasma, urine and bile after oral administration and intravenous injection

No	Retention time (min)	$[M-H]^-$	Assigned identify	Found in[a]				HPLC/ESI–MS^2 (m/z)
				DSS	CA	FA	IFA	
1	10.01	197	Danshensu	U,P/U,P	-/-	-/-	-/-	73(18), 135(6), 153(4), 179(100)
2	12.10	355	3-(3,4-Dihydroxyphenyl) crylic acid glucuronide	-/-	U,P/U,P	-/-	-/-	113(15), 175(14), 179(100), 221(5), 271(4)
3	13.72	213	Danshensu hydroxide	U/U	-/-	-/-	-/-	151(100), 169(55), 195(24)
4	14.45	369	3-(3-Methyoxy-4-hydroxyphenyl) crylic acid glucuronide	-/-	U,B,P/U,B	U,B,P/U,B,P	-/-	113(35), 135(10), 175(100), 179(10), 193(78), 325(7)
5	16.01	211	4-Hydroxy-3-methyloxyphenyl lactic acid	U,P/U	-/-	-/-	-/-	123(5), 165(47), 193(100)
6	16.71	355	3-(3-Hydroxy-4-*O*-glucuronyl) crylic acid	-/-	U,B,P/U,B,P	-/-	-/-	113(47), 135(35), 175(45), 179(100), 293(4), 311(94), 337(5)
7	17.47	259	3-(3-Methyoxy-4-*O*-sulphate-phenyl) crylic acid or 3-(3-*O*-sulphate-4-methyoxy-phenyl) crylic acid	-/-	U/-	-/-	-/-	97(9), 135(4), 179(100), 215(10)
8	17.95	211	3-Hydroxy-4-methyloxyphenyl lactic acid	U/U	-/-	-/-	-/-	134(4), 149(5), 193(100)
9	19.53	369	3-(3-Hydroxy-4-methyoxyphenyl) crylic acid glucuronide	-/-	U,B,P/U,B	U,B,P/U,B	U,B,P/U,B,P	113(46), 175(40), 193(100), 351(5)
10	19.83	179	Caffeic acid	-/-	U,B,P/U,B,P	-/-	-/-	135(100)
11	20.03	259	3-(3-Methyoxy-4-*O*-sulphate-phenyl) crylic acid or 3-(3-*O*-sulphate-4-methyoxy-phenyl) crylic acid	-/-	U/-	-/-	-/-	97(9), 135(4), 179(100), 215(10)
12	20.19	355	3-(3-*O*-Glucuronyl-4-hydroxy) crylic acid	-/-	U,P/-	-/-	-/-	113(100), 135(46), 175(30), 179(97), 267(7), 311(24), 337(41)
13	20.85	369	3-(3-Methyoxy-4-*O*-glucuronyl-phenyl) crylic acid	-/-	U/U	U,P/U	-/-	113(37), 131(9), 175(24), 193(100), 259(12)
14	21.59	369	3-(3-Hydroxy-4-*O*-glucuronyl-phenyl) crylic acid methyl ester/3-(3-*O*-glucuronyl-4-hydroxy-phenyl) crylic acid methyl ester	-/-	U/-	-/-	-/-	113(18), 175(20), 179(4), 193(100), 324(4)
15	22.84	369	3-(3-*O*-Glucuronyl-4-methyoxy-phenyl) crylic acid	-/-	U,B,P/-	-/-	U,B,P	113(84), 161(15), 175(20), 193(100), 296(23), 325(12), 351(15)
16	27.13	211	3,4-Dihydroxyphenyllactic acid methyl ester	U,P/U	U,B/U,P	-/-	-/-	156(6), 165(100), 182(3), 193(2)
17	27.46	193	Ferulic acid	-/-	U/-	U,B,P/U,B,P	-/-	74(18), 134(7), 149(100), 178(55)
18	28.81	193	Isoferulic acid	-/-	-/-	-/-	U/U,B,P	134(10), 149(100), 178(50)
19	29.75	211	3-(3,4-Dihydroxyphenyl)-2-methyoxypropionic acid	U,P/-	U/U	-/-	-/-	123(9), 167(100), 184(5)

[a] Found in: U, urine; P, plasma; B, bile; -, not found; /, stands for oral administration/intravenous injection.

found in the feces. The HPLC–MS^n data of those metabolites were shown in Table 1.

3.2.1. Analysis of metabolites after dosing of danshensu

Quasi-molecular ion $[M-H]^-$ of M1 was 197, and product ions were 73, 135, 153, 179. Both of the retention time and the product ions of M1 were consistent with that of DSS standard. So M1 was assigned as DSS.

There are four hydroxyl groups in the structure of DSS, and four methylated conjugates with quasi-molecular ion $[M-H]^-$ 211 were detected: M5, M8, M16 and M19. We supposed that they were the four *O*-methylated conjugates of DSS on the four hydroxyl groups. The most abundant product ion of both M5 and M8 were 193, and the MS^3 of m/z 193 $[M-H-H_2O]^-$ was 178 $[M-H-H_2O-CH_3]^-$, 149 $[M-H-H_2O-CO_2]^-$, 134 $[M-H-H_2O-CO_2-CH_3]^-$. The fragments of m/z 193 were exactly the same as those of FA and IFA. Because there was a methoxyl group on the phenyl ring of FA and IFA, we could deduce that M5 and M8 were the metabolites of DSS after methylation occurred on one of the hydroxyl groups on the phenyl ring, so M5 and M8 were 3-hydroxy-4-methyloxy-phenyl lactic acid or 4-hydroxy-3-methyloxy-phenyl lactic acid. Comparing with the polarities of FA and IFA, we supposed that M5 might be 4-hydroxy-3-methyloxyphenyl lactic acid and M8 might be 3-hydroxy-4-methyloxyphenyl lactic acid. Product ions of M16 were 156, 165, 182 and 193. We deduced that m/z 193 was the fragment ion after losing the hydroxyl group on the side chain, and then the methoxyl group on the carboxyl could be rearranged through a six-member ring transition state to be connected with the C-2 on the benzene ring, and CO was lost to form the fragment ion m/z 165. Hence M16 should be 3,4-dihydroxyphenyl lactic acid methyl ester. For M19, the product ion with maximum intensity was 167 $[M-H-CO_2]^-$, and the MS^3 of m/z 167 was 123 $[M-H-CO_2-CHOCH_3]^-$. No product ion of m/z 193 could be found, and M5 and M8 have been identified as methylated DSS on the phenolic hydroxyl group, indicating that the hydroxyl group might not be on the side chain, therefore M19 could be 3-(3,4-dihydroxyphenyl)-2-methoxypropionic acid.

M3 was also found in urine after dosing of DSS. The Quasi-molecular ion $[M-H]^-$ of M3 was 213, and a series of product ions at m/z 195 $[M-H-H_2O]^-$, 169 $[M-H-CO_2]^-$, and 151 $[M-H-CO_2-H_2O]^-$ were found. The $[M-H]^-$ of M3 was 16 larger than that of DSS and they shared the same fragmentation pathway, thus M3 was assigned as hydroxylated danshensu.

As shown in Table 1, prototype, methylated conjugates and hydroxylated metabolites (M1, M3, M5, M8, M16 and M19) were found after dosing of DSS. Previous research was mainly focused

on the metabolism after dosing of Danshen extract or compound Danshen prescription. Wei et al. did not find any metabolites of phenolic acids in rat after dosing of crude drug [18]. Zheng et al. reported one metabolite with quasi-molecular ion $[M-H]^-$ 377, and another with quasi-molecular ion $[M-H]^-$ 239. They assigned the two metabolites as danshensu monoglucuronide and isopropylated danshensu [13]. However, quasi-molecular ion $[M-H]^-$ of danshensu monoglucuronide was actually 373, and they failed to find any product ions of glucuronide, so we doubted that it was not the product metabolized by uridine-diphosphate-glucuronosyltransferase (UGT) in rat liver or intestine. Meanwhile, it was not reasonable to identify 239 to isopropylated danshensu, and Pei et al. ever reported it to be acetylated danshensu [12]. We also found both of metabolites in our research, but both peaks were very low and they could also be found in blank samples. Zheng et al. also reported one peak with quasi-molecular ion $[M-H]^-$ 313 and proposed that it might be sulfated danshensu [19,20], but in our research we did not find the same peak. The inconsistence of previous report indicated that metabolic research on DSS after dosing of compound Danshen prescription or Danshen crude drug had its deficiency. Other constituents in compound prescription or crude drug might interfere with the metabolism of certain phenolic acid monomers, for most of the water soluble constituents in Danshen could be considered as polymers constructed by small molecules like DSS, CA and IFA, and these polymers might be decomposed in body [16,21]. In this study, we were trying to conduct the research from a new perspective by comparing the metabolism after dosing of single compound. According to Ross and Wootton, benzoic acids with a free phenolic group inhibited the enzymes to the cerebral metabolism, and greatest effect occurred when there were two hydroxyl groups on the benzene ring. However, methylated conjugates showed no activity [22]. In our research, methylated conjugates were the main metabolites after dosing of danshensu through oral administration or intravenous injection. Prototype (M1) could be found both after injection and oral administration, and hydroxide conjugate could only be found in urine. Because we found neither danshensu nor its metabolites in bile or feces, and the methylated metabolites could not be found in plasma after intravenous injection, this indicated that the First Pass Effect in liver produced methylated conjugates which were then absorbed into blood. Because of the high solubility in water, it was difficult for DSS and its metabolites to be reabsorbed into renal tubule, and they could be excreted solely via kidney.

3.2.2. Analysis of metabolites after dosing of caffeic acid

Quasi-molecular ion $[M-H]^-$ of M10 was 179 with product ion 135. Both of the retention time and the product ions of M10 were consistent with that of CA standard. So M10 was assigned as the prototype of CA.

Quasi-molecular ion $[M-H]^-$ of M17 was 193, and product ions were 134, 149, 178. Because both of the retention time and the product ions of M17 were consistent with that of FA standard, it was assigned as the prototype of FA.

Quasi-molecular ion $[M-H]^-$ of M4, M9, M13, M14 and M15 was 369. Fragment ions of 113, 175, which were product ions of glucuronide [23,24], could be found in MS^2, showing that they should be methylated caffeic acid monoglucuronides. There are three hydroxyl groups in CA, so 6 different methylated caffeic acid monoglucuronides could be produced. FA and IFA could be considered as methylated CA on the phenolic hydroxyl (see Fig. 1), hence the comparison of the difference after dosing of each compound could be helpful to determine the bending sites of methyl and glucuronyl groups. M4 and M13 could only be found after dosing of CA and FA, therefore the methoxyl group should be connected at the C-3 of the benzene ring. For M13 m/z 325 $[M-H-CO_2]^-$ could be found in the product ions, so the glucuronide was connected with 4-hydroxy and M13 should be 3-(3-methoxy-4-*O*-glucuronyl-phenyl) crylic acid while M4 was 3-(3-methoxy-4-hydroxy-phenyl) crylic acid glucuronide. M14 could only be found in CA samples, so the methyl group was not connected to the phenolic hydroxyl. It should be 3-(3-methoxy-4-*O*-glucuronyl-phenyl) crylic acid methyl ester or 3-(3-*O*-glucuronyl-4-methoxy-phenyl) crylic acid methyl ester. M9 and M15 could be found after dosing of IFA or CA. For M15 m/z 325 $[M-H-CO_2]^-$ could also be found in the product ions, so the glucuronide was connected with 3-hydroxy and M15 was 3-(3-*O*-glucuronyl-4-methoxy-phenyl) crylic acid while M9 should be (3-hydroxy-4-methoxy-phenyl) crylic acid glucuronide.

Quasi-molecular ion $[M-H]^-$ of M2, M6 and M12 was 355. The MS^2 of all these metabolites included 113 and 175, indicating that they are caffeic acid monoglucuronide. Besides m/z 113 and 175, the product ions of M6 included 135 $[M-H-Glucuronide-CO_2]^-$, 179 $[M-H-Glucuronide]^-$, 293 $[M-H-H_2O-CO_2]^-$, 311 $[M-H-CO_2]^-$, 337 $[M-H-H_2O]^-$, and for M12, m/z 337 $[M-H-H_2O]^-$ and m/z 267 $[M-H-H_2O-CHCHCO_2]^-$ could be found in the product ions, indicating that the glucuronide group was not connected with carboxyl group in M6 and M12. By comparing the polarities with FA and IFA, we proposed that M6 might be 3-(3-hydroxy-4-*O*-glucuronyl) crylic acid, and M12 might be 3-(3-*O*-glucuronyl-4-hydroxy) crylic acid. The m/z 179 was the most abundant product ion of M2, indicating that the glucuronide connected to the carboxyl was much easier to be broken in the mass spectra, and M2 should be 3-(3,4-dihydroxyphenyl) crylic acid glucuronide.

Two metabolites (M7 and M9) with Quasi-molecular ion $[M-H]^-$ 259 were identified as sulfated CA. Fragment ion m/z 179 was the Quasi-molecular ion losing a sulfate group, and the fragment of m/z 97 could also be found in the MS^2 as $O-SO_3H$.

Two metabolites with Quasi-molecular ion $[M-H]^-$ 211 were also found after oral administration or intravenous injection of CA. We had identified them as M16 and M19, the hydroxylated products after methylation.

For CA, one methylated conjugate (M17), three glucuronized conjugates (M2, M6, M12), five glucuronized conjugates after methylation (M4, M9, M13, M14, M15), two methylated conjugates after hydroxylation (M16, M19) and two sulfated conjugates (M7, M11) could be found besides the prototype CA (M10). Peppercorn et al. reported that methylation was the only reaction in germ-free rats after dosing of CA [25]. According to our results, methylated metabolites were the major products in liver, although hydroxylation was found as a Phase I reaction before methylation occurred. Previous study showed that *O*-methylation is a detoxification route and a preferred pathway for caffeic acid hepatic metabolism [25–27]. Our results indicated that CA was partly hydroxylated in liver, then the product coupled with most of the prototype were methylated in liver firstly, and enthrohepatic circulation might exist so the methylated conjugates could be reabsorbed. These metabolites and the prototype were further metabolized by the intestinal flora by UGT to become glucuronized conjugates in intestine. It seemed that methylated conjugates could not be further sulfated in intestine, because we only detected sulfated CA in urine samples.

3.2.3. Analysis of metabolites after dosing of ferulic acid and isoferulic acid

For FA, Quasi-molecular ion $[M-H]^-$ of M17 was 193, and we have identified it as FA standard. Meanwhile, three glucuronized conjugates M4, M9 and M13 were also found after dosing of FA.

For IFA, Quasi-molecular ion $[M-H]^-$ of M18 was 193, and product ions were 134, 149, 178. Both of the retention time and the product ions of M18 were consistent with those of IFA standard. So M18 was assigned as the prototype of IFA. Meanwhile, two

glucuronized conjugates M9 and M15 were also found after dosing of IFA.

FA and IFA were actually *O*-methylated CA on one of the phenolic hydroxyl groups, and all metabolites found in FA and IFA samples could be found in CA samples. We only found mono-methylation in CA samples and none of methylated conjugates after dosing FA or IFA, so methylation did not occur in FA and IFA samples. Conjugation with glucuronic acid was the only reaction, and conjugation on the carboxyl group on the side chain (M4 and M9, respectively) was the main metabolic pathway after dosing of FA or IFA. It was interesting to note that 3-(3-hydroxy-4-methyoxyphenyl) crylic acid glucuronide (M9) could also be found FA samples. We speculated that M9 with the structure 3-hydroxy-4-methoxy-phenyl was more stable than M4 with the structure 3-methyoxy-4-hydroxy-phenyl, so M4 could be transformed to M9 through a transition state with a five-membered ring.

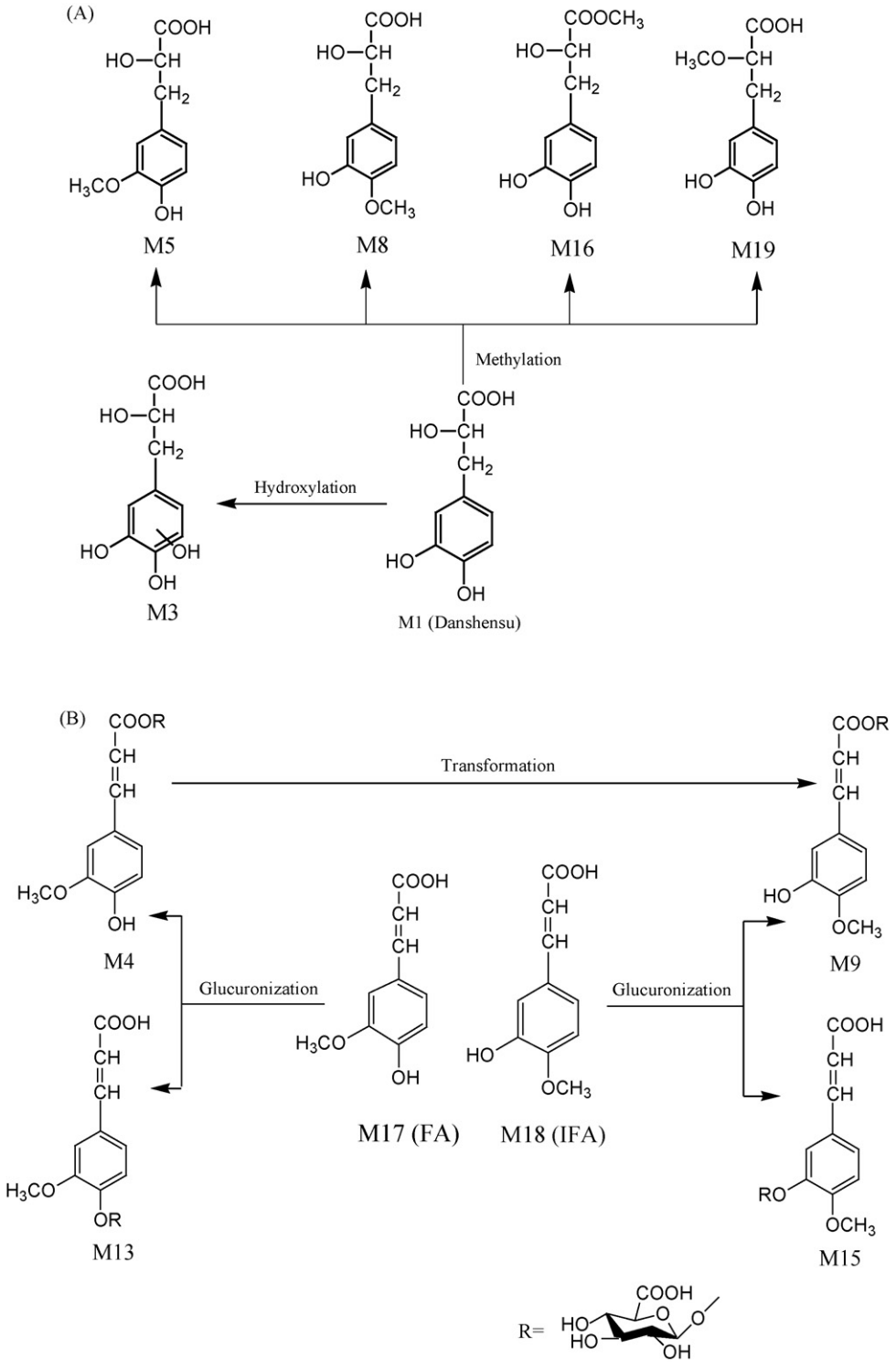

Fig. 3. Proposed metabolic pathway of DSS, CA, FA, and IFA in rats. (A) Metabolic pathway of DSS, (B) metabolic pathway of FA and IFA, and (C) metabolic pathway of CA.

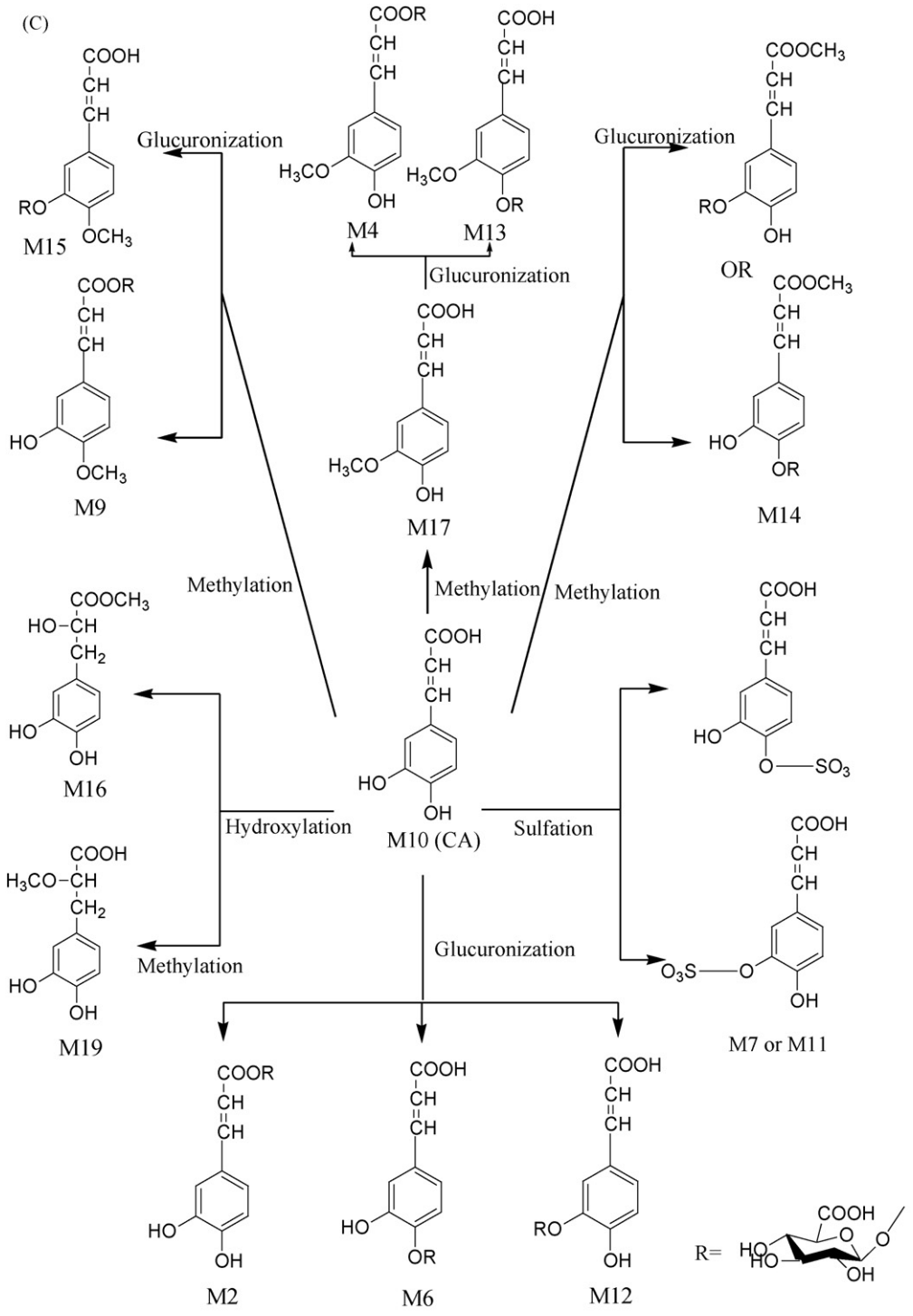

Fig. 3. (*Continued*).

3.3. Proposed metabolism pathway and rules after dosing of phenolic acid monomers

Drug metabolism in body could be divided into two phases. Phase I reactions include oxidations, reductions and hydrolyse, and biotransformation in this phase is the prerequisite for subsequent metabolism. Polar groups like hydroxyl, amino, carboxyl and thiol were formed, and drugs were activated or inactivated in this phase. During Phase II step, conjugations occurred on the polar groups catalyzed by transferases and accelerated drug excretion. For DSS, CA, FA and IFA, totally one primary metabolite (Phase I) and fourteen secondary metabolites (Phase II) were found after dosing of phenolic acid monomers. Prototype and Phase II metabolites were the majority after drug dosing, indicating that for these water soluble drugs it was possible that they were excreted from kidney without transformation, and the polar groups could be catalyzed by transferases directly to form the Phase II metabolites [10,28,29].

By analyzing the metabolism after dosing of single compound which shared the similar structures, we were able to make a comparison and propose the metabolic pathway of DSS, CA, FA, and IFA. Fig. 3 showed the proposed metabolic pathway of the four compounds. Through the comparison of the four phenolic acids, we explored the influence of structure on drug metabolism and disposition. Lafay et al. reported that absorbed phenolic acids are poorly excreted in the bile or into gut lumen [30], and we did not find danshensu or its metabolites in rat bile. However, if dehydration occurred on the side chain and a double bond was formed, like CA, FA and IFA, then both of the prototype and the metabolites could be found in bile. For example, while M16 could only be found in urine and plasma after dosing of DSS, it was found in bile in CA samples. Besides, various secondary metabolites which could not be observed in DSS samples, especially the glucuronized conjugates, were found after dosing of CA, FA or IFA. These result demonstrated that the existence of double bond on the side chain was crucial for the distribution and excretion pathway of these phenolic acid monomers. We supposed that the alteration resulted from the reduction of polarity, and the alteration further changed the metabolism by enriching the type of metabolites.

For FA and IFA, a methoxyl group existed on the benzene ring. Comparing the metabolism of FA and IFA with that of CA, only the prototype and glucuronized could be observed after dosing of FA or IFA. Since methylation was the only Phase II reaction in liver [25], this result indicated that the prototype could only be partly metabolized to methylated conjugates, and prototype could be further metabolized to glucuronized and sulfated conjugates, while only glucuronization occurred for methylated conjugates. Methylation was not found in intestinal metabolism. Although Danshen recipe had been commonly used in clinical therapy, the rules of metabolism and the origination of metabolites were still unclear. This work provided a basis to explain the metabolic pathway and disposition of phenolic acid monomers in Danshen, and helped further explore on drug design for better pharmacokinetic parameters and bioactivity.

4. Conclusion

A new HPLC–ESI-MS^n method coupled with liquid–liquid extraction has been established for the analysis of DSS, CA, FA, IFA and their metabolites in rat urine, bile and plasma, respectively. The method was simple, sensitive and reliable. Totally 19 metabolites including the prototype were found after oral administration or intravenous injection. They are hydroxylated, methylated, sulfated and glucuronized conjugates. In this study, we initiated the investigation of the metabolism and disposition of phenolic acid monomers from Danshen by dosing of single compound. By comparing the metabolism of different compounds, we found that methylation occurred firstly in liver, but further metabolism of methylated conjugates was inhibited except glucuronization. The double bond on the side chain in CA, FA and IFA was critical for the drug distribution in bile and enriches the type of metabolites. This study provided scientific basis for further studies on the measurement of the bioactivity of the metabolites and structure modification for drugs with better bioactivity and pharmacokinetic parameters.

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