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Structural characterization of metabolites of salvianolic acid B from *Salvia miltiorrhiza* in normal and antibiotic-treated rats by liquid chromatography–mass spectrometry

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Abstract

This study was conducted to compare the *in vivo* metabolites of salvianolic acid B (Sal B) between normal rats and antibiotic-treated rats and to clarify the role of intestinal bacteria on the absorption, metabolism and excretion of Sal B. A valid method using LC–MS^n analysis was established for identification of rat biliary and fecal metabolites. And isolation of normal rat urinary metabolites by repeated column chromatography was applied in this study. Four biliary metabolites and five fecal metabolites in normal rats were identified on the basis of their MS^n fragmentation patterns. Meanwhile, two normal rat urinary metabolites were firstly identified on the basis of their NMR and MS data. In contrast, no metabolites were detected in antibiotic-treated rat urine and bile, while the prototype of Sal B was found in antibiotic-treated rat feces. The differences of *in vivo* metabolites between normal rats and antibiotic-treated rats were proposed for the first time. Furthermore, it was indicated that the intestinal bacteria showed an important role on the absorption, metabolism and excretion of Sal B. This investigation provided scientific evidence to infer the active principles responsible for the pharmacological effects of Sal B.

Keywords: Salvianolic acid B; *In vivo* metabolites; LC–MS^n; Intestinal bacteria; Antibiotic-treated rats

1. Introduction

The dried root of *Salvia miltiorrhiza* (Danshen), a commonly used traditional Chinese medicine, is widely used to treat coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis and chronic renal failure [1–4]. Salvianolic acid B (Sal B) is the most common constituent in *Salvia* species and the most abundant in their aqueous extracts [5]. Different *in vitro* studies showed that Sal B has various biological activities of anti-oxidation, anti-inflammatory, anti-hypoxic and anti-arteriosclerotic [6–11]. It is assumed in these *in vitro* studies that Sal B reaches the target inner tissues as bioavailable component. However, Sal B is poorly absorbed through the gut barrier because of their high molecular weight [12,13]. It is possible that these biological effects may not be due to a direct action of Sal B itself but due to an effect of some more readily absorbed low-molecular-weight metabolites. Therefore it is essential to understand how Sal B is absorbed, metabolized and eliminated from the body. So far, attention has been focused on the pharmacokinetics, tissue distribution, metabolism and biliary excretion after intravenous administration of Sal B from the roots of *Salvia miltiorrhiza* [14–20]. In some studies, Sal B revealed extremely low bioavailability and four methylated metabolites were excreted rapidly into rat bile after oral dosing [5,12]. Our previous study mainly focused on the urinary metabolites after oral administration of total phenolic acids [21]. However, to our knowledge, there has been no report with regard to the identification of rat urinary and fecal metabolites after oral dosing of Sal B.

In traditional Chinese medicine, most of the remedies are administered orally in the form of crude decoction and active components have to cross the intestinal barrier to reach the systemic circulation. Active components of their prescriptions are therefore brought into contact with bacterial flora

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in the alimentary tract [22]. Large spectrum of antibiotics have the potential to alter the content of intestinal bacteria and may interfere with the pharmacokinetics and eventually pharmacodynamics of compounds [23], therefore germ-free or antibiotic-treated animals can be used to determine the role of the intestinal microflora in metabolism of foreign compounds *in vivo*. The publication has shown the co-administration of kampo medicines with antibiotics or bacterial preparations [24]. Recently, the correlation of intestinal flora and activity of kampo ingredients has increasingly been recognized. Xing et al. reported the interaction of baicalin and baicalein with antibiotics in the gastrointestinal tract [25]. However, no report has been found in the literature to discuss the effect of antibiotic treatment on the absorption, metabolism and excretion of Sal B.

In recent years, liquid chromatography/mass spectrometry (LC/MS) has been proved to be a powerful and reliable analytical approach for structural analysis of chemical components in herbal extracts with high sensitivity and low consumption of samples [26–29]. Furthermore, tandem mass spectrometry techniques have been playing an important role in metabolic study, such as the structural elucidation of drug metabolites, because the high sensitivity of MS as an LC detector facilitates the discovery of new active constituents which are difficult to obtain by conventional means [30,31]. Eventually, LC–MS^n technique has been applied in this study to investigate the metabolites in rat biosamples after oral dosing of Sal B.

In the present study, we proposed the possible metabolic pathway of Sal B and showed that the intestinal bacteria played an important role on the absorption, metabolism and excretion of Sal B. Two urinary metabolites, four biliary metabolites and five fecal metabolites of Sal B were detected in normal rat biosamples by isolating the pure compounds or using LC–MS^n technique. In contrast, no metabolites were detected in antibiotic-treated rat urine and bile, while the prototype of Sal B was found in antibiotic-treated rat feces. For the first time, we investigated the differences of *in vivo* metabolites between normal rats and antibiotic-treated rats after oral dosing of Sal B. Two urinary and five fecal metabolites in normal rats were firstly identified in biosamples. This finding provided a scientific basis from a metabolic point of view for the clarification of action mechanism of Sal B.

2. Experimental

2.1. Materials and chemicals

Sal B was isolated from the roots of *Salvia miltiorrhiza* by the author and the purity was not less than 95% by comparison with the analytical standard of Sal B via a reverse-calculation procedure. The analytical standard of Sal B was purchased from Sikehua Biotech Co. Ltd. (Sichuan, China).

HPLC-grade acetonitrile was purchased from Caledon, Canada. HPLC-grade water was prepared using a Milli-Q water purification system (Bedford, MA, USA). Methanol, ethyl acetate, trifluoracetic acid and formic acid in mobile phase were AR grade, purchased from Beijing Chemical Corporation (Beijing, China). Oasis® HLB extraction cartridges were purchased from Waters (Etten-Leur, The Netherlands).

2.2. Animals and drug administration

Male Sprague-Dawley rats (12–14 weeks of ages; 200–240 g body weight) were provided by the Experimental Animal Center, Peking University Health Science Center, China. Animals were kept in an environmentally controlled breeding room for 3 days before starting the experiments. They were fed with food and water *ad libitum* and fasted overnight before urine, feces and bile collections. The protocols of animal experiments were approved by the Animal Center of Peking University Health Science Center. Sal B was dissolved in deionized water (20 mg/ml) and administered by oral gavage at a dose of 100 mg/kg body weight. Deionized water was administered orally to the rats at a dose of 5 ml/kg body weight for blank urine, bile and feces collections.

Antibiotic-treated rats were administered a cocktail of antibiotics according to the method of Kinouchi et al. [32]. A mixture of neomycin sulfate and streptomycin sulfate (1:1, w/w; 200 mg/kg) dissolved in sterilized water was given orally to rats twice daily for 6 days. During that period, the animals had free access to diet and autoclaved distilled water. Animals were fasted overnight before the urine, bile and feces collections. Antibiotic-treated rats received a dose of Sal B (100 mg/kg) by oral gavage, and deionized water was administered orally to the rats at a dose of 5 ml/kg body weight for blank sample collections. The antibiotic treatment was continued for 2 more days after administration of Sal B aliquot or deionized water.

2.3. Sample collection and processing procedures

Urine, bile and feces samples after oral administration of Sal B were collected over 0–12 h, 12–24 h, 24–36 h and 36–48 h periods. All samples were stored at −80 °C until analysis. Blank rat urine, bile and feces samples were collected to check whether they were free of interfering components.

Urine sample was extracted by using Oasis® HLB extraction columns. The column was preconditioned with 6 ml methanol and 6 ml deionized water. The urine sample passed through the column at gravity flow. After washing with 6 ml of deionized water, retained material was washed from the column with 6 ml of MeOH–H_2O (2:3, v/v). The eluate was concentrated to dryness under a flow of nitrogen gas at 35 °C, and reconstituted in 300 μl methanol.

Samples of bile (2 ml) were mixed with 300 μl of 10% (v/v) hydrochloric acid which were then thoroughly vortex-mixed for 2 min, followed by the addition of 6 ml ethyl acetate to each tube. Extraction was performed by vortex mixing the tubes for 5 min. After centrifugation at 9000 rpm for 5 min, the supernatant was transferred to a clean test tube and dried under a flow of nitrogen gas at 35 °C. The residue was reconstituted in 300 μl methanol. After filtering through a membrane (0.45 μm pore size), a 10 μl aliquot was injected into the chromatographic system for analysis.

Feces samples were weighted and supplemented with an appropriate volume (6 ml/g) of the mixture of methanol and water (7:3, v/v). After ultrasonic extraction for 20 min, the samples were centrifuged at 9000 rpm for 5 min. The supernatant was transferred to a clean test tube and dried under a flow of nitrogen gas at 35 °C. The residue was reconstituted in 300 μl methanol. After filtering through a membrane (0.45 μm pore size), a 10 μl aliquot was injected into the chromatographic system for analysis.

2.4. Isolation of urinary metabolites (M1 and M2)

In order to obtain sufficient amounts of M1 and M2 from excretion study urine samples, 400 ml of urine from 16 normal rats was collected over 0–48 h after oral dosing of Sal B and concentrated to 40 ml by using rotary evaporator. The concentrated urine sample was extracted with 10 Waters Oasis® HLB extraction columns. The column was preconditioned with 6 ml of methanol and 6 ml of deionized water. The urine sample was applied in 4 ml aliquots and passed through the column at gravity flow. After washing with 6 ml of deionized water, retained material was washed from the column with 6 ml of MeOH–H_2O (2:3, v/v). The eluate was concentrated to dryness under a flow of nitrogen gas at 35 °C, and reconstituted to 2 ml in methanol. Further repeated column chromatography using Agilent Zorbax Extend C_{18} reversed-phase column, which was eluted with acetonitrile −0.05% trifluoracetic acid, afforded M1 (2 mg) and M2 (4 mg). The mobile phase flow rate was 0.8 ml/min, and the gradient program was used as follows: 5–33% A at 0–40 min and 33–5% A at 40–50 min. The purity of each metabolite was 98% and 96%, respectively, based on HPLC analyses.

2.5. Instrumentation

2.5.1. HPLC

The analyses were performed using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany), equipped with an on-line degasser, a quaternary solvent delivery system, an autosampler, a column temperature controller and a diode-array detector. The analytical column was Zorbax Extend C_{18} column (5 μm, 250 mm × 4.6 mm, Agilent) connected with a Zorbax Extend C_{18} guard column (5 μm, 12.5 mm × 4.6 mm, Agilent). The mobile phase consisted of acetonitrile (A) and 0.1% aqueous formic acid (B). A gradient program was used as follows: 5–33% A at 0–40 min and 33–5% A at 40–50 min. The mobile phase flow rate was 0.8 ml/min and column temperature was maintained at 20 °C. UV spectra were recorded from 190 to 400 nm and the detection wavelength was set at 288 nm.

2.5.2. Mass spectrometry

For HPLC–MS analysis, a Finnigan LCQ Advantage ion trap mass spectrometry (Thermo Finnigan, San Jose, CA, USA) was connected to the Agilent 1100 HPLC system via an ESI interface. The LC effluent was introduced into the ESI source in a post-column splitting ratio of 2:1. Ultra-high purity helium (He) was used as the damping and collision gas, and high purity nitrogen (N_2) was used as the sheath and auxiliary gas. For negative ESI analysis, the optimized parameters were as follows: sheath gas, 45 arbitrary units; auxiliary gas, 5 arbitrary units; spray voltage, 4.5 kV; capillary temperature, 350 °C; capillary voltage, −30 V; tube lens offset voltage, 20 V. For full-scan MS analysis, the spectra were recorded in the range of *m/z* 100–1000. Data-dependent acquisition was used so that the two most abundant ions in each MS scan were selected in turn. The relative collision energy for CID was adjusted at 40–45% of maximum to acquire satisfactory product ion spectra, and the isolation width of precursor ions was 3.0 Th.

3. Results and discussion

3.1. Optimization of extraction, HPLC and HPLC–MS^n methods

According to the literatures, Li et al. investigated the extraction of serum performed by acetone and ethyl acetate [15–18], and Liu et al. also reported that 70% methanol was selected as the extraction solvent because of its highest extraction efficiency of the six phenolic acids [33]. Therefore, solid phase extraction (SPE) with Waters Oasis® HLB and MAX cartridges, liquid–liquid extraction (LLE) with different solvents (ethyl acetate, methanol, the mixture of ethyl acetate and acetone) and ultrasonic extraction (USE) with 70%, 100% methanol and ethyl acetate had been tried. We added the Sal B standard into the biosamples before extraction procedure. And the recoveries of the extraction method were calculated after the procedure. As to urine sample, SPE with MAX could not provide as much information of metabolites as SPE with HLB. LLE with ethyl acetate and SPE with HLB showed higher recoveries (67.06% for LLE with ethyl acetate and 51.08% for SPE with HLB) than LLE with methanol (16.75%) as illustrated in Fig. 1A. However, SPE with HLB could provide more satisfactory information of urinary metabolites. Eventually, we selected SPE with Waters Oasis® HLB cartridge to extract the urine sample. In regard to bile samples, LLE with ethyl acetate and methanol, SPE with HLB had been tried. As shown in Fig. 1B, LLE with ethyl acetate was finally selected with the highest recovery of 73.18% and SPE with HLB could not provide the more information of biliary metabolites this time. Meanwhile, the recoveries of SPE with HLB and LLE with methanol were 50.50% and 2.95%, respectively. Furthermore, considering the unstable property of fecal metabolites, we choose ultrasonic extraction and 70% methanol was finally selected according to the literature [33]. Fig. 1C showed the chromatograms of different extraction solvents (70% methanol, 100% methanol and ethyl acetate) for feces sample. In conclusion, SPE with Waters Oasis® HLB cartridges, LLE with ethyl acetate and ultrasonic extraction with 70% methanol were chosen to extract the urine, bile and feces samples, respectively, since it could ensure the simultaneous extraction of the most target compounds and least interference from the co-eluted endogenous matrixes.

To obtain chromatograms with good separation, stationary phase, mobile phase, column temperature, flow rate and detection wavelength were investigated. For the assay of phenolic acids, the stationary phase of Zorbax Extend C_{18} column was

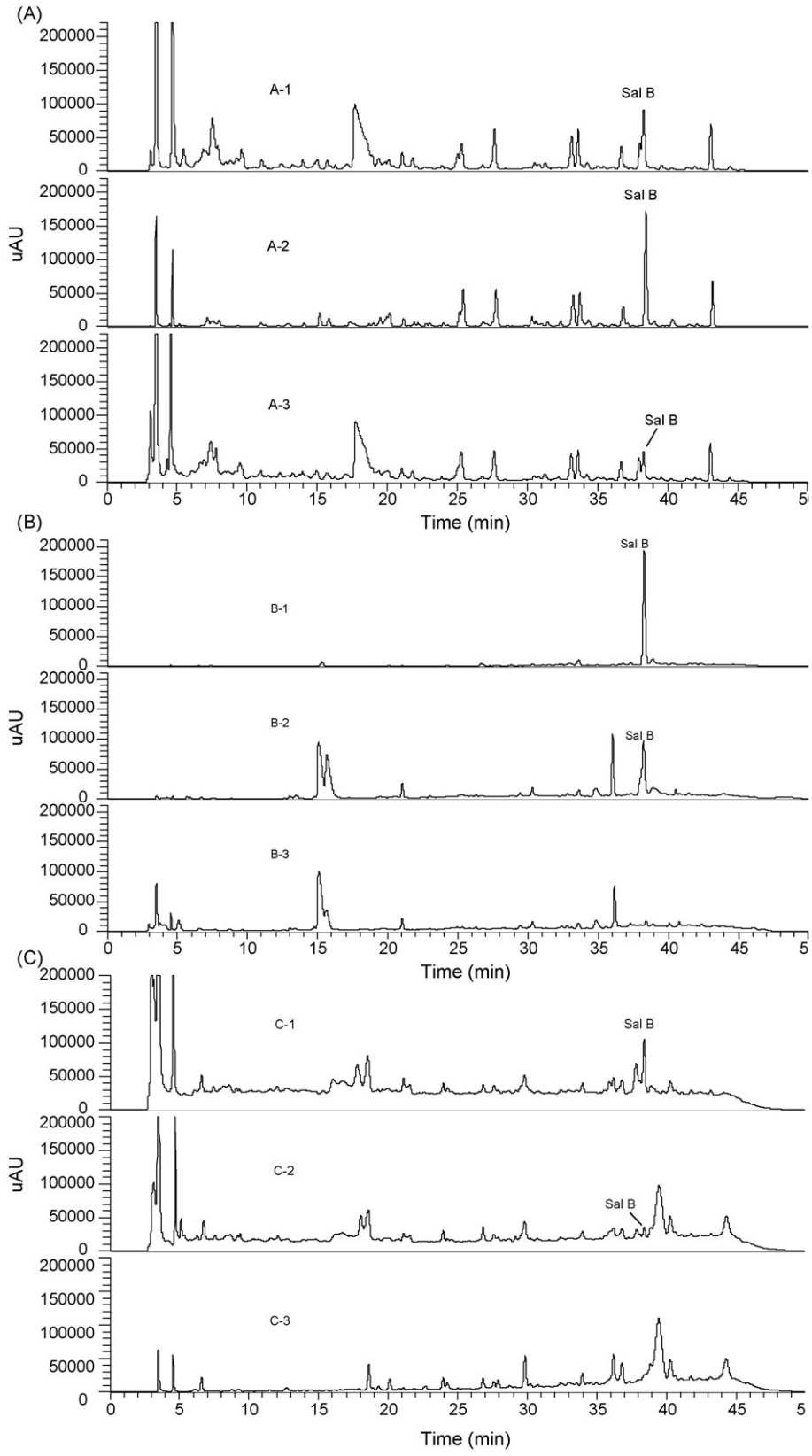

Fig. 1. The chromatograms of the various extraction method to biosamples. (A) Blank urine sample spiked with Sal B standard; A-1: SPE with HLB; A-2: LLE with ethyl acetate; A-3: LLE with methanol. (B) Blank bile sample spiked with Sal B standard; B-1: LLE with ethyl acetate; B-2: SPE with HLB; B-3: LLE with methanol. (C) Blank feces sample spiked with Sal B standard; C-1: USE with 70% methanol; C-2: USE with 100% methanol; C-3: LLE with ethyl acetate.

better than that of YMC-Pack ODS-A C_{18}, Phenomenex Luna C_{18}, Alltech Alltima HP C_{18} and Zorbax SB C_{18} columns. MeCN–H_2O gave better separation than MeOH–H_2O, which was therefore selected as the mobile phase. 0.1% (v/v) HCOOH was added to the mobile phase. It was also found that the best separation was achieved when the column temperature was kept at 20 °C using a flow rate of 0.8 ml/min. Diode-array detector (DAD) was applied to select the optimized wavelength of Sal B and its biosamples, and the HPLC chromatogram at 288 nm showed more peaks and better separation than that at other wavelengths. Table 1 showed the symmetry factor (*S*), theoretical plate number (*N*) and resolution (*R*) of these differences among above changes. Considering the features of chromatograms, the R.S.D. of the retention times of the five metabolites identified in the normal rat feces samples after oral administration of Sal B were used to evaluate the developed HPLC method. The HPLC method precision was measured by analyzing the same sample for five consecutive times. The R.S.D. of the five peak retention times were less than 1.51%. To test the stabilities of the analytes, the same sample was analyzed in 2 days. Peak retention time R.S.D. for all of the five peaks were less than 1.86%, suggesting that the sample remained stable within 2 days. All of above results of precision and stability test indicated that this method was sensitive, reliable and applicable.

ESI in both negative and positive ion modes were tried and the results showed that ESI in negative ion mode was more sensitive and could produce more fragmentation information for Sal B in the present study. The instrumental parameters were optimized by analyzing the Sal B standard for the maximum intensity. The optimized parameters in the negative ion mode were as follows: ion spray voltage, 4.5 kV; sheath gas, 45 arbitrary units; auxiliary gas, 5 units; capillary temperature, 350 °C; capillary voltage, −30 V; tube lens offset, 20 V. To obtain the most abundant information for all the constituents in rat biosamples, the data-dependant scan was used in LC–MS^n analysis. The two most abundant ions in each scan were selected and subjected to MS^n analyses and the relative collision energy for CID was set at 45%, which could produce the satisfactory MS^n fragmentation information.

3.2. Fragmentation pathway of Sal B

In order to identify the metabolites, Sal B was firstly analyzed by direct injection. As illustrated in Fig. 2, the negative electrospray mass spectrum of Sal B showed a $[M-H]^-$ ion at *m/z* 717. The ESI-MS/MS spectra of the $[M-H]^-$ ion exhibited fragments derived from neutral loss of two molecules of Danshensu (DSS) (198 u) or caffeic acid (CA) (180 u), resulting in the fragment ions at *m/z* 537 (-CA), 519 (-DSS), 339 (-DSS-CA) and 321 (-DSS-DSS), but it is difficult to determine the dissociation sequence of each of the DSS and CA moieties from the side chain of the furan ring or of the phenyl ring. In addition, the tendency of losing DSS is greater than that of losing CA for tetrameric phenolic acids derivatives as Zeng et al. mentioned [34]. This can be exemplified as the abundance of ions at *m/z* 519 (−198 u, 100%) is larger than that of *m/z* 537 (−180 u, 2%), and the abundance of *m/z* 321 (−198 u −198 u, 18%) is larger than

Table 1
Symmetry factor (*S*), theoretical plate number (*N*) and resolution (*R*) of the different HPLC method for normal rat 12–24 h feces sample after oral administration of Sal B

Stationary phase	Mobile phase	Temperature (°C)	Flow rate (ml/min)	M7			M8			M9			M10			M11		
				S	*N*	*R*	*S*	*N*	*R*	*S*	*N*	*R*	*S*	*N*	*R*	*S*	*N*	*R*
Extend	$CH_3CN-HCOOH$	20	0.8	0.77	8755	5.09	1.52	21554	1.28	1.07	83725	1.54	0.97	129122	3.41	0.94	181074	1.31
Extend	$CH_3CN-HCOOH$	20	1.0	0.00	12175	2.29	1.59	2988	0.60	0.97	47534	5.92	1.06	99159	2.91	0.88	118878	0.78
Extend	$CH_3CN-HCOOH$	25	0.8	2.75	11852	3.46	1.28	7048	1.20	1.06	44699	3.36	0.96	105280	1.48	0.98	121120	1.14
Extend	$CH_3CN-HCOOH$	30	0.8	1.41	8049	3.71	0.96	6635	1.08	1.03	33223	4.96	1.12	109967	1.69	1.11	141739	1.52
Extend	$CH_3CN-HAc$	20	0.8	0.00	29136	2.86	0.95	9623	0.82	0.99	56594	5.16	0.78	83338	6.59	0.60	95856	0.55
YMC	$CH_3CN-HCOOH$	20	0.8	–	–	–	0.89	25614	2.04	0.83	112080	1.69	0.86	175133	1.99	0.84	192433	1.84
Luna	$CH_3CN-HCOOH$	20	0.8	0.00	31039	8.57	0.97	30021	0.55	0.93	119543	6.00	0.74	159951	2.54	0.84	181847	1.66
Alltima	$CH_3CN-HCOOH$	20	0.8	0.00	20165	5.86	1.89	15570	1.42	1.08	70505	3.19	1.33	128584	2.59	0.90	168761	1.05
SB	$CH_3CN-HCOOH$	20	0.8	0.00	34466	7.24	1.28	8438	0.81	1.01	53283	5.32	0.84	1222628	2.46	0.84	127644	0.95

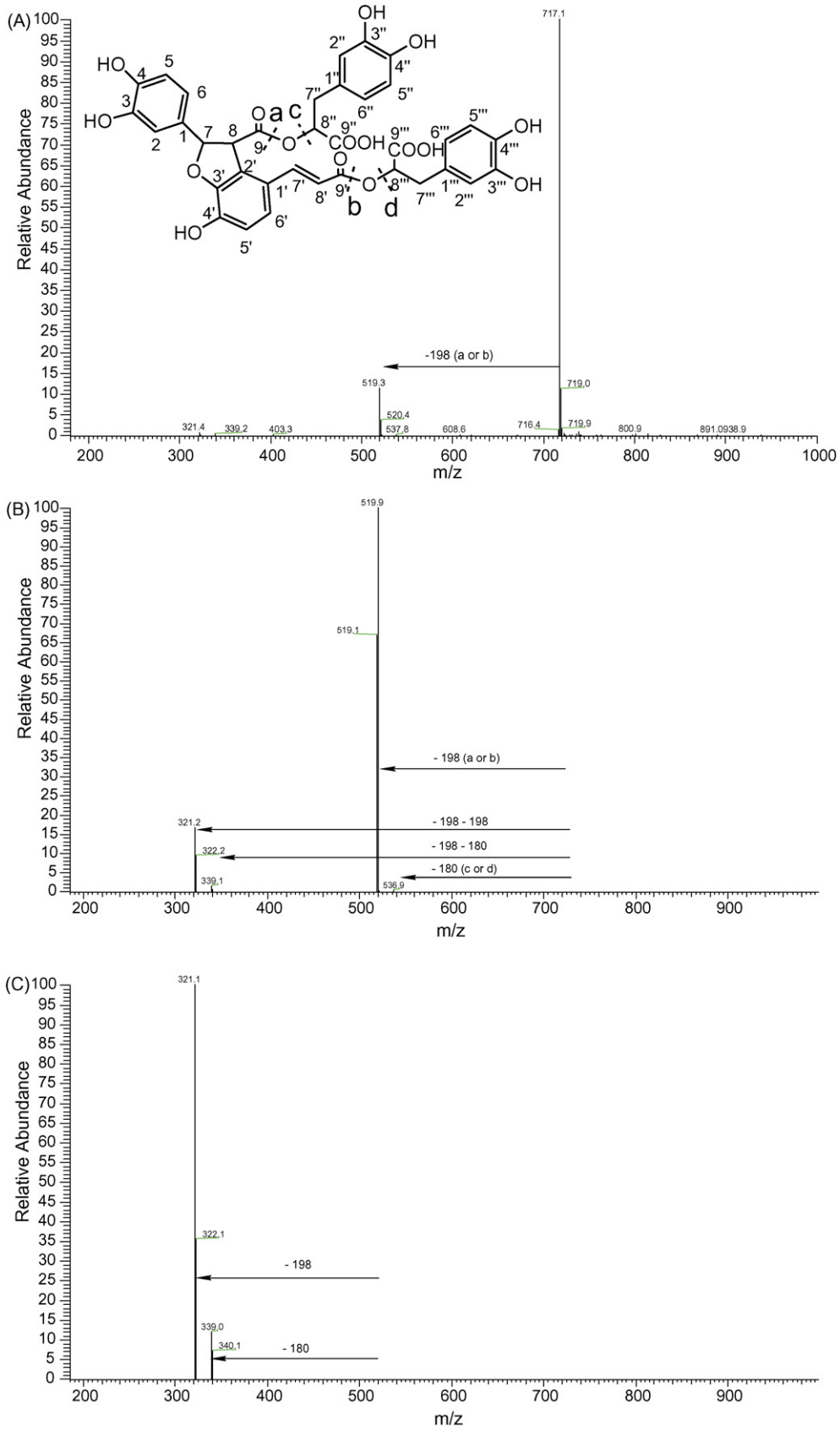

Fig. 2. MS^n ($n = 1$–4) fragmentation of Sal B standard. (A) MS; (B) MS^2 [717]; (C) MS^3 [717 → 519]; (D) MS^4 [717 → 519 → 321].

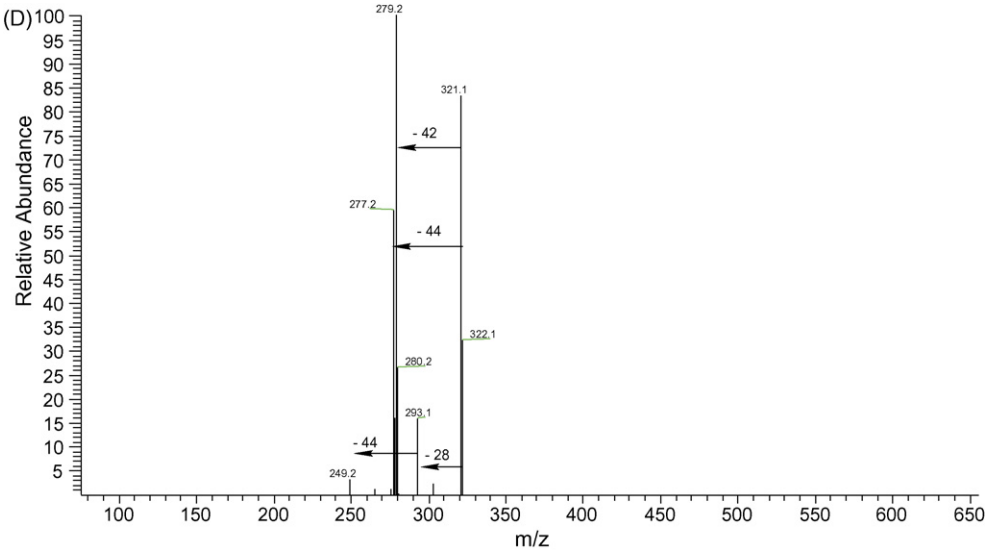

Fig. 2. (*Continued*).

that of *m/z* 339 (−198 u −180 u, 3%), in the CID spectrum of Sal B. According to the literature [34], except for prolithospermic acid, all components of dimeric, trimeric and tetrameric phenolic acids in *S. miltiorrhiza* showed the same general fragmentation pattern for their $[M-H]^-$ ions, involving losses of DSS (−198 u) and CA (−180 u) via dissociation of the bonds on either side of the esterifiable carboxyl oxygen. And the tendency of losing DSS is more easily than CA. In the MS^3 spectrum of Sal B, there were two prominent ions: $[M-H-198-198]^-$ ion at *m/z* 321 (100%) and $[M-H-198-180]^-$ ion at *m/z* 339 (13%) corresponding to the loss of the second DSS and the first CA. In the MS^4 spectrum of Sal B, the ions of $[M-H-198-198-CO]^-$ (*m/z* 293) and $[M-H-198-198-CH_2-CO]^-$ (*m/z* 279) could be observed. The fragmentation pathway of Sal B was proposed and applied for identification of compounds with phenolic acid skeleton in rat biosamples.

3.3. Urinary metabolites of Sal B in normal rats and in antibiotic-treated rats

Under the established LC conditions, LC chromatograms of urine samples from normal rats were shown in Fig. 3. By comparing the LC chromatograms of blank urine samples from normal rats with that of urine samples after oral dosing of Sal B, it was found that two urinary metabolites (M1 and M2), which isolated from the procedure described above, occurred in the normal rat urine samples over 0–48 h after administration. And no difference existed in the antibiotic-treated rat urine sample after administration by comparing with the blank antibiotic-treated rat urine sample.

By comparing the MS and NMR data of M1 with literature [35], M1 showed the same properties as 3-(3-hydroxyphenyl)-propionic acid. MS data for M2, another compound isolated from normal rat urine samples after dosing of Sal B, showed a prominent $[M-H]^-$ product ion at 204, which was assigned to the molecular ion. Subsequently, this fragment ion expels 44 Da (–COOH) as proved in MS^2 experiment producing the characteristic ion at *m/z* 160. Moreover, by comparing the NMR data of M2 with literature [36], M2 was identified as *trans*-cinnamoylglycine.

3.4. Biliary metabolites of Sal B in normal rats and in antibiotic-treated rats

3.4.1. HPLC/MS chromatograms of rat bile samples

In order to identify the biliary metabolites, the possible structures of metabolites had been speculated according to the metabolism rule of drugs firstly [5]. The metabolites (M3–M6) were observed in the normal rat bile samples in comparison with the blank bile sample and elucidated by electrospray LC–MS^n using a combination of full and product ion scanning techniques. The UV and total ion current (TIC) chromatograms of blank and normal rat bile samples were shown in Fig. 4. The profile of normal rat bile sample was greatly different from that of blank bile sample, which illustrated the changes of Sal B in the course of *in vivo* physiological disposition. On the other hand, no metabolites were found in the antibiotic-treated rat bile samples after oral dosing of Sal B.

3.4.2. Identification of biliary metabolites in normal rats

The structures of four metabolites in normal rat bile were elucidated by a combined analysis of the UV, MS and MS^n spectra. Table 2 showed the HPLC–DAD–MS^n data of four metabolites in normal rat bile. The UV data of M3–M6 showed the characteristic of phenolic acids according to the literature [37]. As shown in Fig. 5, the negative ESI-MS spectra of M3 showed the $[M-H]^-$ ion at *m/z* 759, 42 u heavier than that of Sal B, suggesting that M3 might be trimethyl Sal B. The CID spectra of the $[M-H]^-$ ion of M3 contained fragment ions at *m/z* 565, 547, 353 and 335, corresponding to $[M-H-194]^-$, $[M-H-212]^-$, $[M-H-212-194]^-$ and $[M-H-212-212]^-$, respectively. The mass loss of 194 u and 212 u are 14 u larger than 180 u and 198 u, respectively, so it is reasonable to assign the 194 and 212 neutral losses as a molecule of the methyl ester

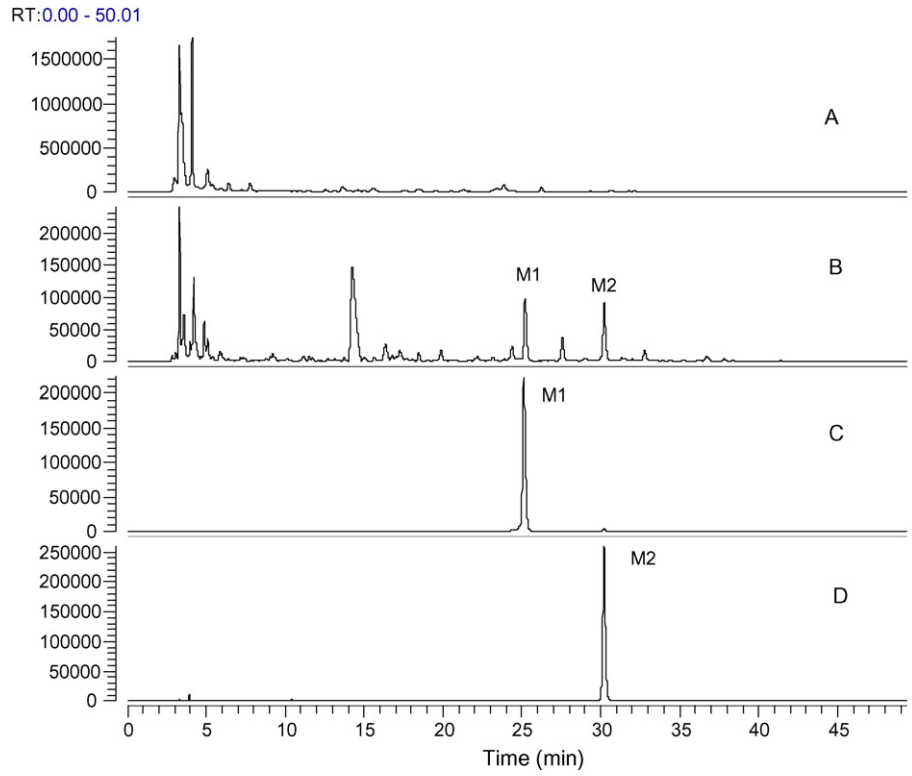

Fig. 3. UV chromatograms of normal rat urine samples. (A) Blank urine sample; (B) 0–12 h urine sample after oral administration of Sal B; (C) M1 standard; (D) M2 standard.

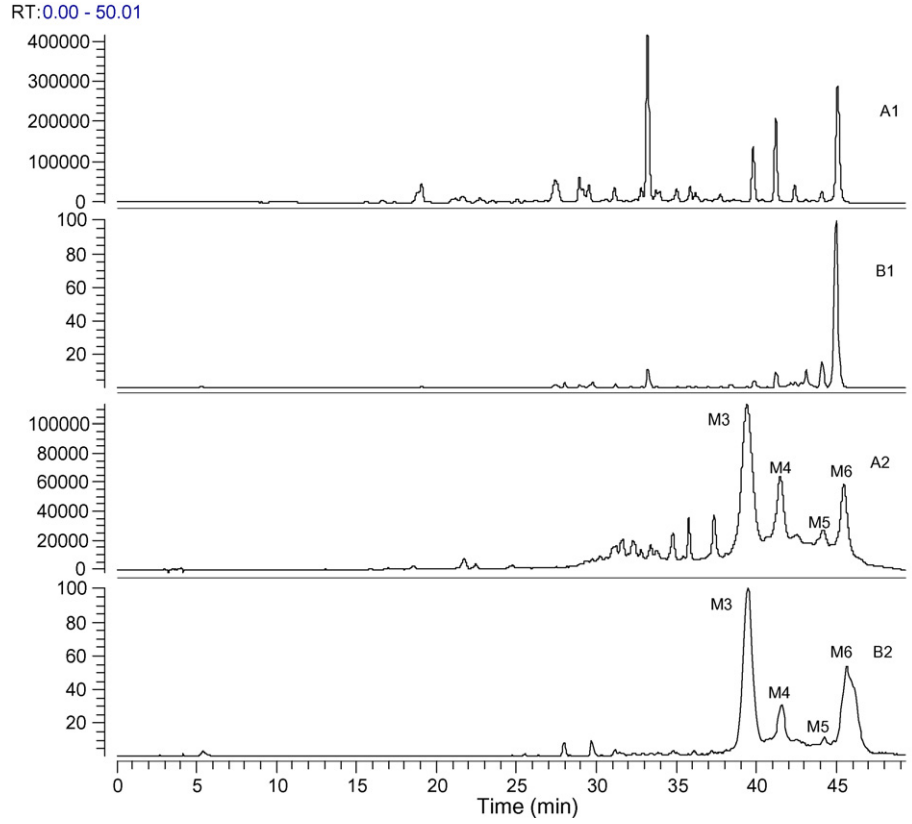

Fig. 4. UV chromatograms (A) and total ion current chromatograms (B) of blank bile sample (1) and 12–24 h bile sample after oral administration of Sal B (2) in normal rats.

Table 2
HPLC–DAD–MS^n data and identification of biliary metabolites of Sal B in normal rats

Peak no.	Retention time (min)	Assigned identify	$[M-H]^-$ *m/z*	UV λ_{max} (nm)	HPLC/ESI-MS^n *m/z* (%base peak)
M3	39.48	3,3″,3‴-*O*-tri-methyl-Sal B	759	286	MS^2[759]: 335(8); 353(6); 547(100); 565(2) MS^3[759 → 547]: 321(5); 335(100); 353(65) MS^4[759 → 547 → 335]: 321(100)
M4	41.56	3-*O*-monomethyl-Sal B	731	286	MS^2[731]: 533(100)
M5	44.24	3,3″-*O*-dimethyl-Sal B or 3,3‴-*O*-dimethyl-Sal B	745	286	MS^2[745]: 533(100) MS^3[745 → 533]: 335(100); 353(13)
M6	45.66	3,3″-*O*-dimethyl-Sal B or 3,3‴-*O*-dimethyl-Sal B	745	286	MS^2[745]: 335(3); 533(10); 547(100) MS^3[745 → 547]: 335(100); 353(45) MS^4[745 → 547 → 335]: 321(100)

of CA and DSS. In addition, it is indicated that three methyl moieties in the structure of M3 should be derived from two side chains of the furan ring and one side chain of phenyl ring by analyses of its MS^n fragmentation. On this basis, M4 was ascribed to 3-*O*-monomethyl-Sal B, while M5 and M6 were identified as either of 3,3″-*O*-dimethyl-Sal B or 3,3‴-*O*-dimethyl-Sal B by studying their MS^n fragmentations and by comparing with literature. However, the current data cannot distinguish M5 and M6. In addition, this work confirmed the investigation of Zhang et al. [5]. And the innovation of our study is to compare the *in vivo* biliary metabolites between the normal rats and the antibiotic-treated rats.

3.5. Fecal metabolites of Sal B in normal rats and in antibiotic-treated rats

3.5.1. HPLC/MS chromatograms of rat feces samples

In the LC–MS chromatogram of the normal rat feces sample, large peaks were detected at 9.36 min (M7), 9.79 min (M8), 20.45 min (M9), 28.77 min (M10) and 30.51 min (M11) by comparing them with the chromatogram of blank feces sample. Furthermore, five metabolites detected in the normal rat feces could not be found in the antibiotic-treated rat feces after oral dosing of Sal B. However, a large amount of M12 was observed in the antibiotic-treated rat feces samples. This is the first time to elucidate the fecal metabolites in normal rats and in antibiotic-treated rats. The LC–MS chromatograms of normal rat and antibiotic-treated rat feces samples are shown in Fig. 6.

3.5.2. Identification of fecal metabolites in rats

In order to identify the structures of metabolites in rat feces after oral administration of Sal B, normal rat and antibiotic-treated rat feces samples obtained 12–24 h after oral dosing were analyzed by the established HPLC/MS^n method. On the basis of full-scan results, specified precursor ions in each MS scan were selected in turn and subjected to tandem mass spectrometry (MS^n, $n = 2$–4) analyses. Table 3 illustrated the HPLC–DAD–MS^n data of five metabolites in normal rat feces.

M8 was designated to be DSS, by comparing the retention time and mass spectra with those of reference compound. Mean-

Table 3
HPLC–DAD–MS^n data and identification of fecal metabolites of Sal B in normal rats

Peak no.	T_R (min)	Assigned identify	$[M-H]^-$ *m/z*	HPLC/ESI-MS^n *m/z* (%base peak)
M7	9.36	(*s*)-3-(3,4-dihydroxyphenyl) lactic acid	197	MS^2[197]: 179(100)
M8	9.79	Danshensu	197	MS^2[197]: 179(100)
M9	20.45	Salvianolic acid R[a]	361	MS^2[361]: 195(8); 221(30); 239(100); 257(5); 273(10); 317(30) MS^3[361 → 221]: 177(100)
M10	28.77	Salvianolic acid S[a]	539	MS^2[539]: 175(18); 201(10); 219(15); 255(2); 271(8); 297(100); 315(2); 341(5); 359(70); 399(95); 417(7); 495(3) MS^3[539 → 399]: 173(5); 179(3); 201(75); 219(100) MS^3[539 → 297]: 161(15); 163(7); 175(100); 187(5); 238(3); 253(15); 269(10); 279(15); 297(60) MS^4[539 → 399 → 219]: 175(100)
M11	30.51	Salvianolic acid T[a]	539	MS^2[539]: 173(2); 175(15); 201(10); 219(18); 271(3); 297(75); 341(2); 359(48); 399(100); 417(2); 495(2); 521(2) MS^3[539 → 297]: 163(15); 175(100); 238(5); 253(25); 255(18); 279(13); 297(75) MS^4[539 → 297 → 175]: 147(100)

[a] Named by the author.

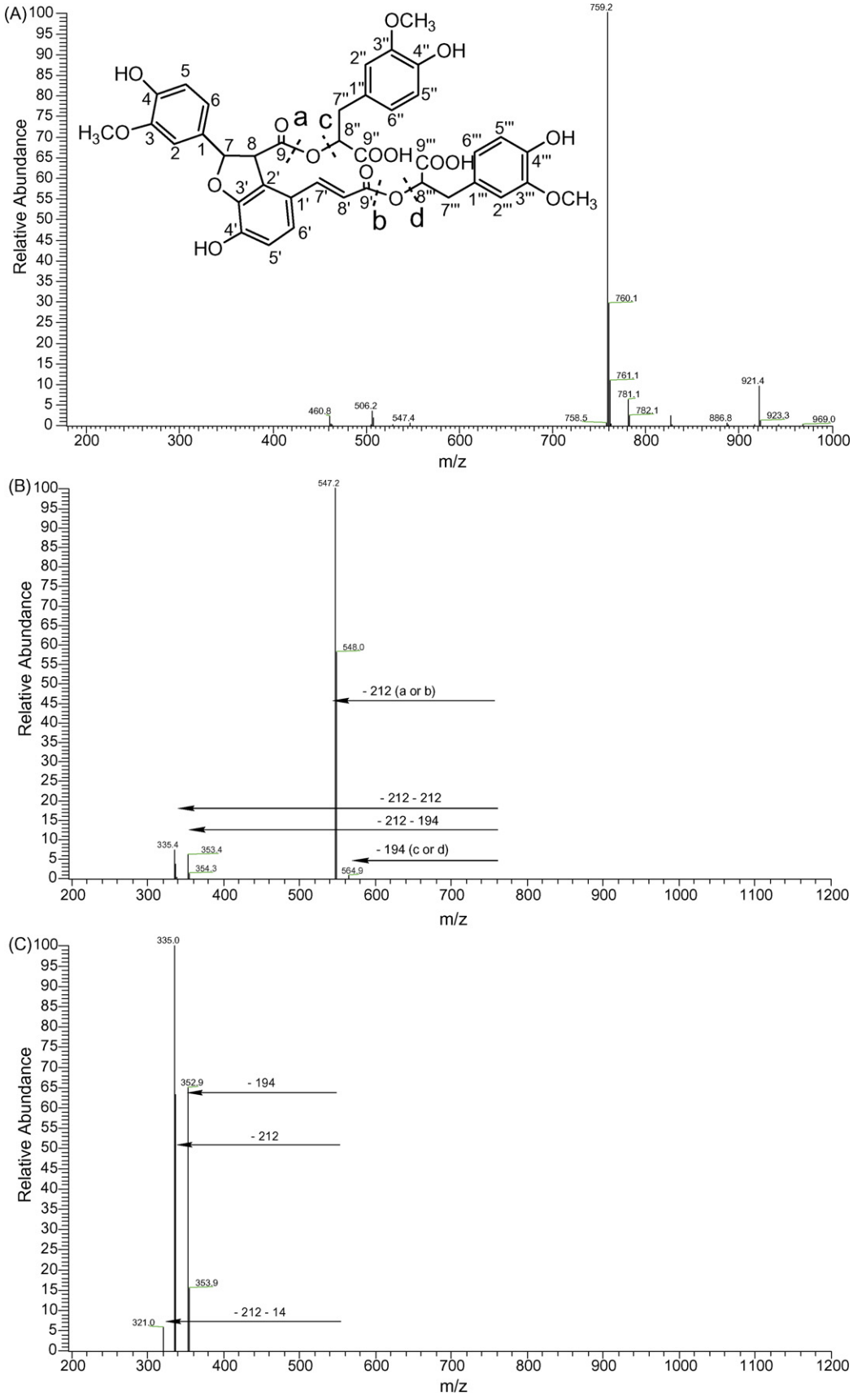

Fig. 5. MS^n ($n = 1$–4) fragmentation of M3. (A) MS; (B) MS^2 [759]; (C): MS^3 [759 → 547]; (D) MS^4 [759 → 547 → 335].

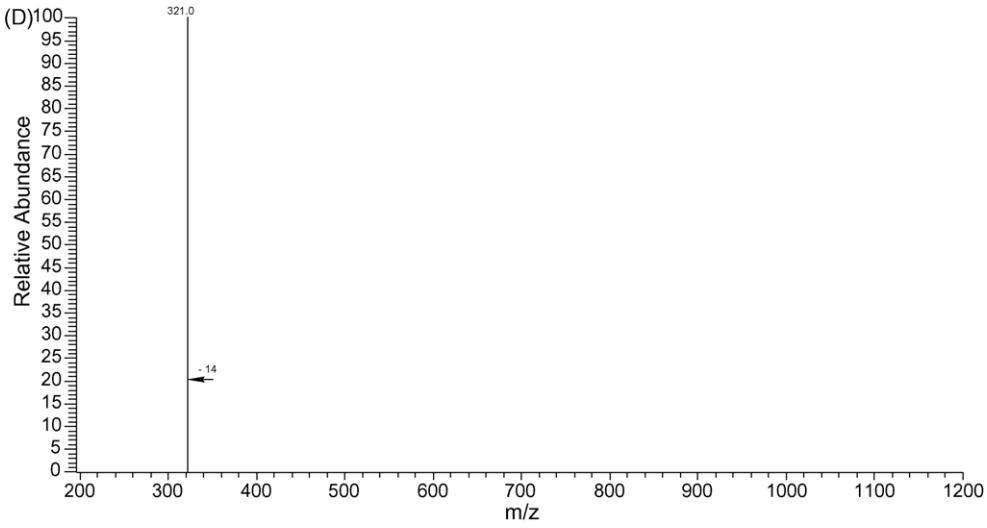

Fig. 5. (*Continued*).

while, the peak at 9.36 min (M7) gave the same $[M-H]^-$ ion and fragmentations as M8. Compared with M8, the peak was tentatively identified as the (*s*)-3-(3,4-dihydroxyphenyl) lactic acid [37]. The UV absorbances of M9–M11 were similar with those of phenolic acids as Liu et al. investigated [38]. And considering M9–M11 as fecal metabolites of Sal B, it was speculated that the metabolites were derived from the bond cleavages of Sal B. The CID spectra of M9–M11 revealed a characteristic fragmentation. The ESI-MS/MS spectra of the $[M-H]^-$ ions of these three metabolites all exhibited fragments derived from neutral loss of 122 u. For example, the CID spectra of the $[M-H]^-$ ions of M9 (*m*/*z* 361), M10 and M11 (*m*/*z* 539) exhibited ions at *m*/*z* 239 (100%) and 417 (7% in M10; 2% in M11), respectively. These two ions could lose one molecule of H_2O subsequently,

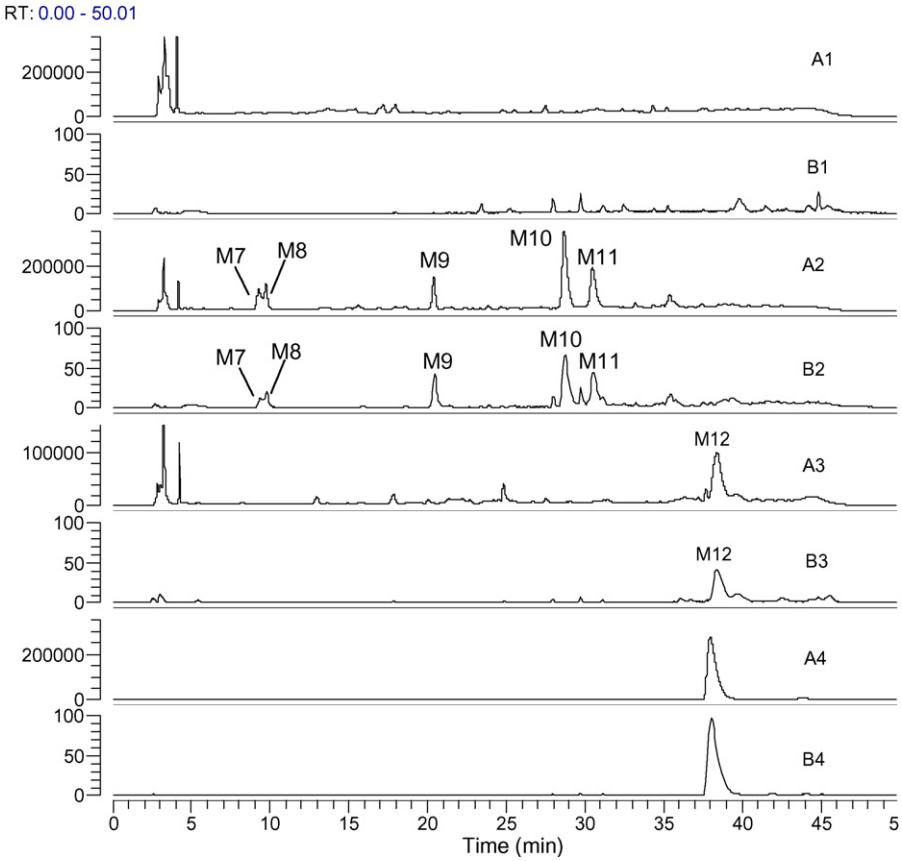

Fig. 6. UV chromatograms (A) and total ion current chromatograms (B) of blank normal rat feces sample (1), 12–24 h normal rat feces sample after oral administration of Sal B (2), 12–24 h antibiotic-treated rat feces sample (3) and Sal B standard (4).

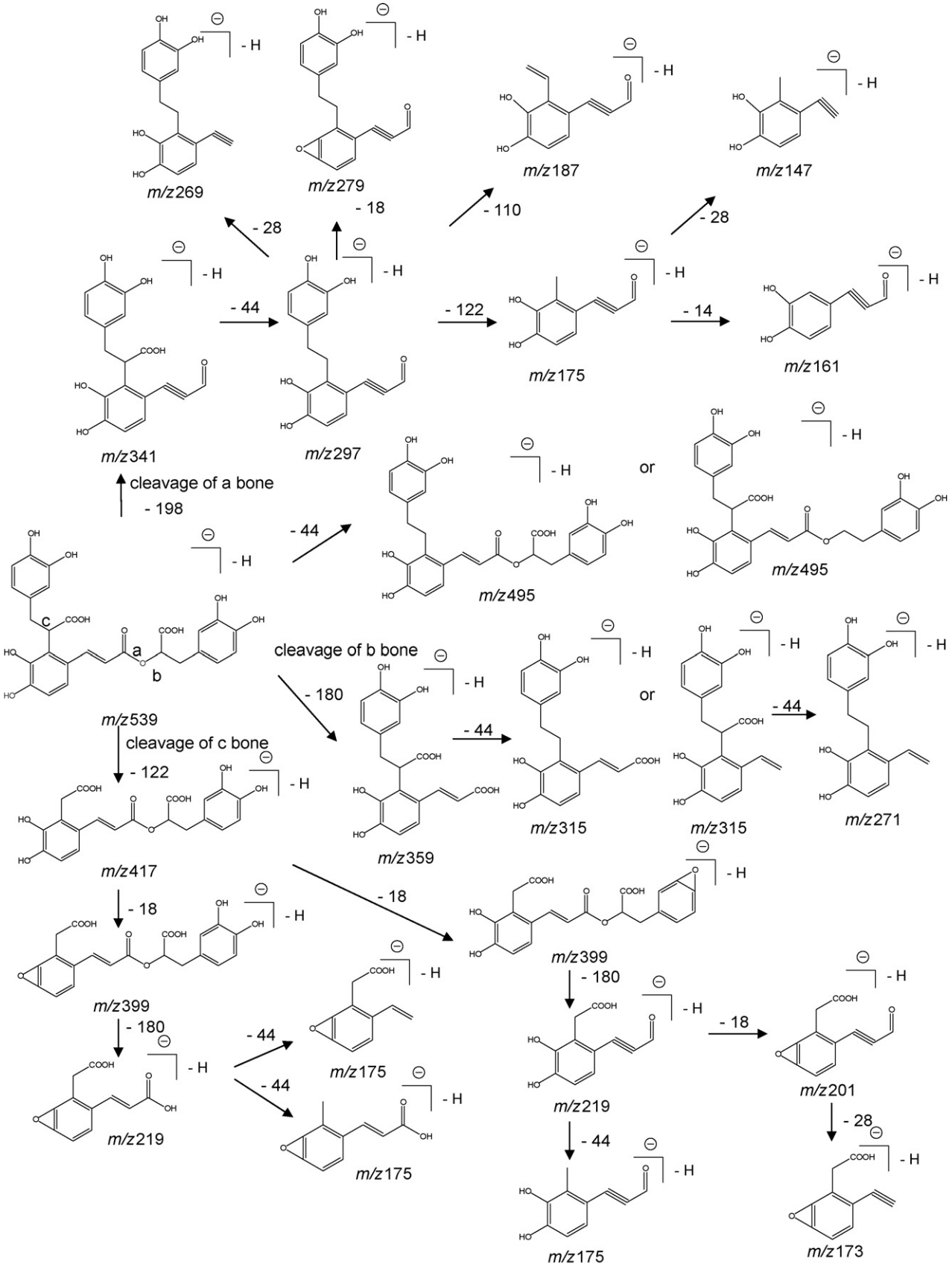

Fig. 7. Proposed MS^n fragmentation pathway of M10 or M11.

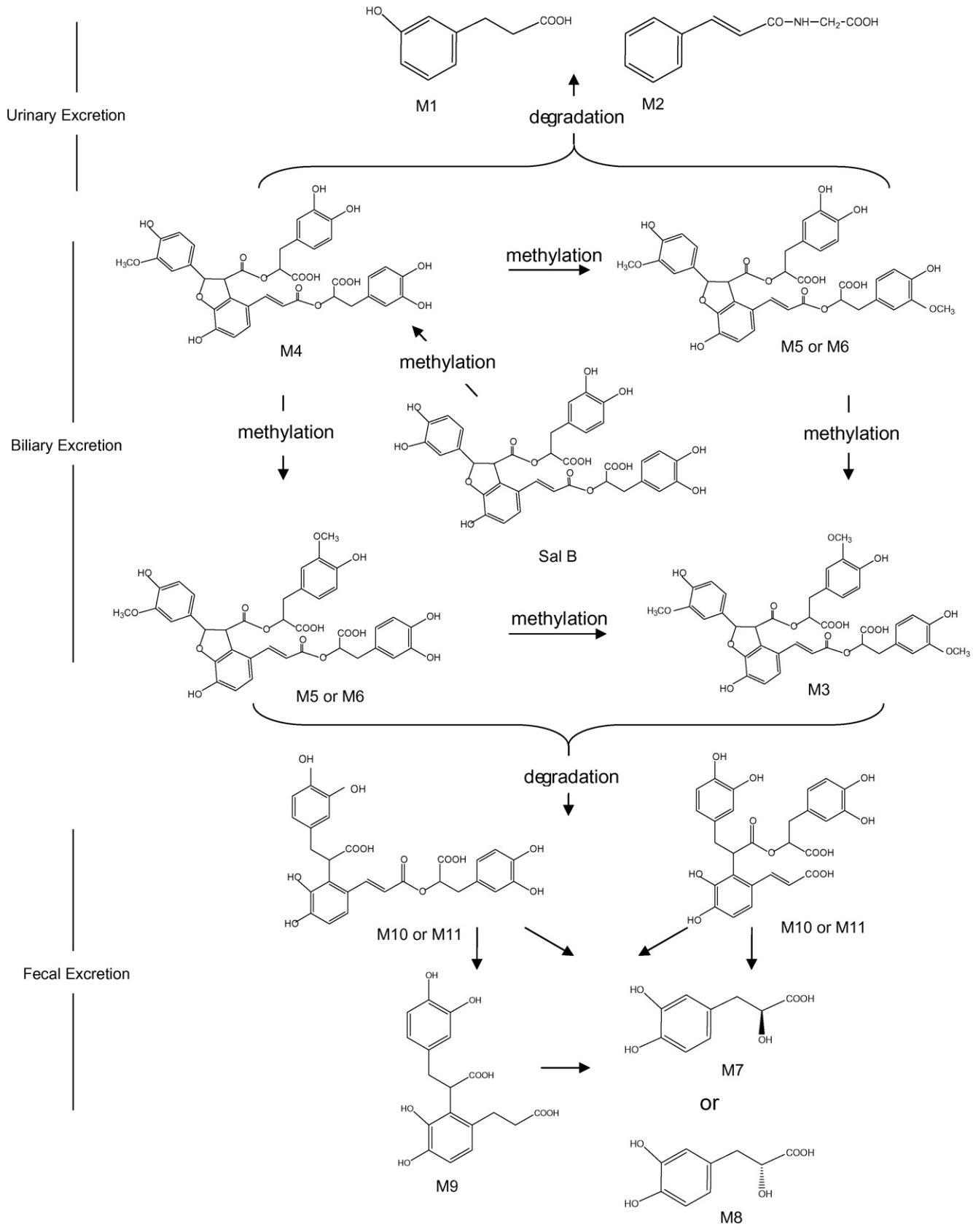

Fig. 8. Proposed metabolic pathways of Sal B in normal rats.

resulting in the fragment ions at *m/z* 221 (30%) and 399 (95% in M10; 100% in M11). According to the literature [39], the dissociation of C ring of flavonoids could be catalyzed by intestinal bacteria. Therefore, it was supposed that the furan ring of Sal B could be dissociated *in vivo* and the characteristic neutral loss of 122 u in M10 was assigned to 3,4-dihydroxyl benzyl moiety by the cleavage of c bond (see Fig. 7). As presented in Fig. 7, one of the fecal metabolites (M10 or M11), of which quasi-molecular ion $[M-H]^-$ was at 539, had been named to be salvianolic acid S. Because both of the two ester bonds in Sal B have the probability to dissociate and expel the same $[M-H]^-$ ion at *m/z* 539 (M10 and M11), another fecal metabolite was therefore ascribed as salvianolic acid T. Meanwhile, M9 was assigned as salvianolic acid R, which originated from the dissociation of all the two ester bonds and furan ring in Sal B. In addition, the cleavages of two ester bonds in Sal B would lead to expel M7 and M8. Consequently, we can conclude that the catalysis by intestinal bacteria should show the effect on furan ring and two ester bonds in Sal B, resulting in the formation of five metabolites. These five fecal metabolites were identified for the first time.

After oral dosing of Sal B, M12 was detected in the antibiotic-treated rat feces samples. The retention time 38.35 min and MS^n fragmentation behavior of M12 was identical to that of Sal B. Hence it was designated as Sal B. The difference of metabolites between the normal rat feces and the antibiotic-treated rat feces was investigated.

3.6. Elucidation of the possible metabolic pathway

The normal rat urine samples after oral dosing of Sal B were collected over 0–12 h, 12–24 h, 24–36 h and 36–48 h periods. M1 and M2 could be detected in the normal rat urine samples of 0–48 h. And in the period of 12–24 h, the amount of the metabolites was highest. According to the literatures [40–45], M1, of which urinary excretion increases after polyphenols administration, was thought to be the microbial metabolism of catechin and proanthocyanidin. We therefore hypothesized that a part of Sal B and its biliary metabolites might largely reach the colon where they can be degraded into various aromatic acid metabolites by the catalysis of microflora. Another compound isolated from the normal rat urine samples after oral dosing of Sal B was *trans*-cinnamoylglycine, the metabolite with the highest content. According to the literatures [46,47], *trans*-cinnamoylglycine should be an endogenous substance in the human urine. However, when Sal B was administered to the rats, the content of the *trans*-cinnamoylglycine was markedly increased. It was speculated that Sal B could produce *trans*-cinnamic acid which was well absorbed from the gastro-intestinal tract. Furthermore, the findings showed that the mouse glycine *N*-acyl-transferase had a high affinity to cinnamic acid which was previously reported as the metabolite of polyphenols [39,45]. Therefore large amount of *trans*-cinnamoylglycine should be detected in the normal rat urine sample after dosing of Sal B. In contrast, M1 and M2 could not be detected in antibiotic-treated rat urine samples after administration of Sal B. It indicated that Sal B should undergo the degradation by rat intestinal bacteria.

As proposed in literature [5], four methylated metabolites of Sal B (one monomethylated Sal B; two dimethylated Sal B; one trimethylated Sal B) could be found in normal rat bile. It was illustrated that catechol *O*-methyl transferase catalyzed the transfer of the methyl group from *S*-adenosyl-*L*-methionine to the *meta*-hydroxyl group of phenolic compounds with a catechol structure. Because Sal B seemed to enter liver cells rapidly where methylated metabolites were produced after oral dosing, these compounds may exert some pharmacological activities in the liver, including antioxidant action. And no existence of metabolites in antibiotic-treated rat bile samples indicated that intestinal bacteria had an important role on the absorption, metabolism and excretion of Sal B. The innovation of our study is to compare the biliary metabolites in normal rat samples with that in antibiotic-treated rat samples.

The fecal metabolites of normal and antibiotic-treated rats after oral dosing of Sal B have been identified for the first time. Our investigation indicated that a part of Sal B absorbed was well excreted into bile as methylated metabolites in normal rats. Subsequently, Sal B and its biliary metabolites were brought into contact with bacterial flora in the alimentary tract. Finally, five degraded metabolites were excreted from the body into the normal rat feces. In the period of 12–24 h, the amount of M10 and M11 was the highest, and it was M9 of which amount was the highest during 24–36 h after oral dosing of Sal B. This result indicated that M9 might be produced by the degradation of M10 and M11. And the dissociations of the furan ring and two ester bonds of Sal B would lead to the production of the five fecal metabolites. The report illustrated that flavonoid and its glycosides could undergo the degradation such as dissociation of the bonds in C ring [39]. However, in contrast, Sal B was excreted into feces as prototype in antibiotic-treated rats and the concentration reached the peak during the period of 24–36 h. The findings suggested that the correlation between antibiotics and Sal B existed, probably because Sal B was mainly metabolized by intestinal bacteria, which was inhibited by antibiotics.

Therefore, as to the normal rat urinary metabolites of Sal B, we could speculate that a part of the degraded fecal metabolites would be easier absorbed and then excreted from the body via rat urine in the form of M1 or M2. The importance of intestinal bacteria in the absorption, metabolism and excretion of Sal B could be increasingly recognized. The possible metabolic pathway of Sal B was proposed in Fig. 8.

4. Conclusions

An HPLC–ESI-MS^n method was developed for the identification of *in vivo* metabolites in normal rat bile and feces after oral administration of Sal B. Meanwhile, the normal rat urinary metabolites of Sal B were identified by isolating the purified compounds. In contrast, no existence of metabolites was detected in antibiotic-treated rat urine and bile, while the prototype of Sal B was found in antibiotic-treated rat feces. For the first time, the differences of *in vivo* metabolites between normal rats and antibiotic-treated rats were investigated. Furthermore, we proposed a possible metabolic pathway of Sal B and found that the intestinal bacteria showed an important

role on the absorption, metabolism and excretion of Sal B. This investigation provided scientific evidence to infer the active principles responsible for the pharmacological effects of Sal B. It was also helpful to better understand the *in vivo* metabolism of Sal B. Further studies are required to identify which of the Sal B metabolites are active in inducing a variety of beneficial physiological functions in animals and humans.

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