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Liquid chromatography–tandem mass spectrometry analysis of protocatechuic aldehyde and its phase I and II metabolites in rat

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Abstract

A method using liquid chromatography–electrospray ionization tandem mass spectrometry (LC–MS/MS) analysis was established for the identification of metabolites in rat after oral administration of protocatechuic aldehyde, a major bioactive phenolic acid in the roots of *Salvia miltiorrhiza*. Eleven metabolites in rat plasma and urine were firstly identified as protocatechuic aldehyde, protocatechuic acid and their methylated, glucuronized or glycine conjugates on the basis of their MS fragmentation behaviors, while nine of these metabolites (except protocatechuic aldehyde and protocatechuic acid) were detected in rat bile. In addition, the possible metabolic pathway was proposed for the first time. In the phase I metabolism, protocatechuic aldehyde could be oxidized to protocatechuic acid. The conjugates would be formed in rat intestine, liver and kidney and excreted from rat urine and bile. Enthrohepatic circulation played an important role in the metabolism of protocatechuic aldehyde. The results proved that the established method was simple, reliable and sensitive, revealing that it could be used to rapid screen and identify the structures of active components responsible for pharmacological effects of protocatechuic aldehyde and to better understand its *in vivo* metabolism.

Keywords: Protocatechuic aldehyde; Metabolites; HPLC–MS^2; *Salvia miltiorrhiza*

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]. Protocatechuic aldehyde (PAL) has been considered as one of the major active constituents of *Salvia miltiorrhiza* [5]. A number of pharmacological studies showed that PAL possessed arrays of biological activities as anti-cardium colic, improving microcirculation, reducing atherosclerosis and inhibiting the aggregation of platelets [6–9]. There are great interests in the therapeutic potential of PAL. Biological properties of PAL closely depend on its bioavailability. Therefore, it is essential to understand how PAL is absorbed, metabolized and eliminated from the body. So far, attention has been focused on the determination of PAL in rat plasma after i.m. Danshen injection or i.g. *Salvia miltiorrhiza* extract [10]. Pan and his coworkers have established an HPLC–DAD–ESI-MS/MS method for the identification of absorbed and metabolic components in rat plasma after oral administration of 'Shuangdan' granule, a commonly used traditional Chinese medicinal preparation made from the aqueous extracts of *Radix Salviae Miltiorrhizae* and *Cortex Moutan*, from which 16 components and 5 metabolites were simultaneously identified [11]. We have previously studied the determination of PAL in rat serum and the metabolism of total phenolic acids in rats after oral administration of *Salvia miltiorrhiza* extract [5,12]. However, to our knowledge, there is no report with regard to the identification of rat metabolites *in vivo* after oral dosing of PAL.

Recently, liquid chromatography/mass spectrometry (LC/MS) has been proved to be a powerful and reliable analytical approach for structural analysis of components in herbal extracts with high sensitivity and low consumption of samples [13–16]. Furthermore, tandem mass spectrometry techniques have been playing an important role in the metabolism study,

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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 [17,18].

In the present study, LC/MS method was adopted to elucidate the structures of 11 metabolites existed in rat plasma, urine and bile on the basis of the mass spectra after oral administration of PAL. Meanwhile, the possible metabolic pathway after oral dosing of PAL was proposed. This investigation provided a basis from a metabolic point of view for the clarification of action mechanism of PAL.

2. Experimental

2.1. Chemicals

PAL was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and the purity was not less than 98% by HPLC analysis.

HPLC-grade acetonitrile was purchased from Caledon, Canada. HPLC-grade water was prepared using a Milli-Q water purification system (Millipore, MA, USA). Ethyl acetate, other chemicals and solvents were all of analytical grade. High-purity nitrogen (99.999%) and helium (99.999%) were purchased from Gas Supplies Center of Peking University Health Science Center (Beijing, China).

2.2. Instrumentation

The analyses were performed using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany), equipped with a diode-array detector which recorded UV spectra in the range of 190–400 nm with the reference wavelength at 600 nm. The column configuration consisted of an Agilent Zorbax Extend C_{18} reversed phase column (5 μm, 250 mm × 4.6 mm, Agilent) and an Agilent 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) with a gradient elution of 5–20% A in 0–35 min and 20–5% A in 35–40 min. The flow rate was 0.8 ml/min and column temperature was maintained at 20 °C. The HPLC chromatogram was monitored at 288 nm for the maximum absorption wavelengthes of PAL metabolites.

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 3:1. Ultra-high purity helium (He) was used as the collision gas and high purity nitrogen (N_2) as the nebulizing gas. For negative ESI analysis, the parameters were as follows: sheath gas, 35 arbitrary units; auxiliary gas, 5 units; spray voltage, 4.5 kV; capillary temperature, 380 °C; capillary voltage, −25 V; tube lens offset, −10 V. For full-scan MS analysis, the spectra were recorded in the range of *m/z* 100–800. Data-dependent acquisition was used so that the two most abundant ions in each MS scan were selected in turn. The collision energy for CID was adjusted at 45% (units specific to ThermoFinnigan systems) to acquire satisfactory product ion spectra, and the isolation width of precursor ions was 3.0 Th.

2.3. Animals and drug administration

Male Sprague–Dawley rats (12–14 weeks of age; 200–240 g body weight) were provided by the Experimental Animal Center, Peking University Health Science Center, China. The animals were maintained at ambient temperature (22–24 °C) and 60% relative humidity with a 12 h light/dark cycle. The protocols of animal experiments were approved by the Animal Center of Peking University Health Science Center. PAL was dissolved in deionized water (5 mg/ml) and administered by oral gavage at a dose of 40 mg/kg body weight. Deionized water was administered orally to the rats at a dose of 10 ml/kg body weight for blank urine and bile collections.

2.4. Collection of samples

Blood samples (1 ml) via the orbital sinus were collected into heparinized tubes immediately before (for blank blood sample) and at 5 min, 15 min, 30 min, 60 min, 90 min and 120 min after oral administration of PAL to two rats, respectively. Plasma was harvested by centrifugation and stored at −80 °C until analysis.

For urine and feces sampling, six rats were divided into two groups and then housed in metabolic cages with free access to deionized water. One group consisting four rats was administered with PAL and the other was administered with deionized water (for blank urine collection). Prior to PAL administration, the rats were deprived of food for 12 h, but had access to deionized water. Urine and feces samples were collected at 10 h and 22 h post-intake. After centrifugation at 9000 rpm for 5 min, all samples were harvested and stored at −80 °C until additional extraction and analysis.

For bile sampling, six rats were fixed on a wooden plate and anesthetized with ether. An abdominal incision was made and the common bile duct was cannulated with PE-10 tubing (ID = 0.08 cm, Becton Dickinson, U.S.A.) for the collection of bile samples, and closed by suturation. PAL was administered to four animals by oral gavage when animals recovered conscious and the other two were administered with deionized water for blank. A heating lamp was used for maintaining the body temperature during the experimental procedure to prevent hypothermic alterations of the bile flow. Bile samples were collected during 0–10 h and 10–22 h periods, and then stored at −80 °C until additional extraction and analysis.

2.5. Sample preparation

Samples of plasma (500 μl), urine (2 ml) and bile (2 ml) were mixed with 80 μl, 300 μl and 500 μl of 10% (v/v) hydrochloric acid, respectively. And then the samples were thoroughly vortex-mixed for 2 min followed by the addition of ethyl acetate in the ratio of 1:3 (v/v) 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 of the mixture of methanol

and 0.1% aqueous formic acid (1:1, v/v). 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.

3. Results and discussion

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

Various sample preparation methods were tested to select an efficient clean-up for biosamples to obtain a better recovery of the target compounds. The methods included liquid–liquid extraction with ethyl acetate and the mixture of ethyl acetate and acetone, and solid-phase extraction with Waters Oasis HLB cartridges. However, solid-phase extraction showed limited extraction efficiency of these analytes, while liquid–liquid extraction exhibited the high recoveries of the spiked standard compounds. Eventually, liquid–liquid extraction with ethyl acetate was chosen because it could ensure the simultaneous extraction of all target compounds. Meanwhile, 10% (v/v) hydrochloric acid was added to achieve the acidity at pH 2.5, so that it could achieve better extraction for phenolic acids.

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 fixed phase of Zorbax Extend C_{18} column was better than that of YMC-Pack ODS-A C_{18}, Phenomenex Luna 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 and detection wavelength of 288 nm [19].

ESI in both negative and positive ion modes were tried and the results showed that ESI in negative ion mode was more sensitive for phenolic acids in the present study. The instrumental parameters were optimized by analyzing the three phenolic acids, protocatechuic acid (PAC), PAL and vanillic acid (VAC) for the maximum intensity. The optimized parameters in the negative ion mode were as follows: ion spray voltage, 4.5 kV; sheath gas, 35 arbitrary units; auxiliary gas, 5 units; capillary temperature, 380 °C; capillary voltage, −25 V; tube lens offset, −10 V. To obtain the most abundant information for all the constituents in rat biosamples, the data-dependent scan was used in HPLC–MS^2 analysis. The two most abundant ions in each scan were selected and subjected to MS/MS analyses and the relative collision energy for CID was set at 45%, which could produce the satisfactory MS^2 fragmentation information.

3.2. In vivo identification of rat metabolites by LC–MS^2

Under the established HPLC conditions, HPLC chromatograms of blank samples and samples of plasma, urine and bile after dosing of PAL are shown in Fig. 1. By comparing the HPLC chromatograms of blank samples with those of samples after oral dosing of PAL, it was found that the profiles of samples after administration were greatly different from those of blank. This illustrated the change of PAL in the course of physiological disposition. In order to screen the absorbed, metabolized and excreted components of PAL in rat, the samples were analyzed by HPLC–MS technique.

In order to identify the metabolites *in vivo*, the possible structures of metabolites had been firstly speculated according to the metabolism rule of drugs [11,20,21]. The full-scan mass spectra of biosamples after administration of PAL were compared with those of blank samples to find out the possible metabolites in rat (see Fig. 1). Then, these compounds were analyzed by HPLC–MS/MS.

Under the aforementioned conditions, 11 metabolites were detected after dosing of PAL. Table 1 shows the HPLC/MS^n data of 11 metabolites in rat plasma, urine and bile. Most of the metabolites in biosamples after dosing of PAL were conjugates. And there are three types of conjugates: methylated, glucuronided and glycine conjugates.

3.2.1. Methylated conjugate

Quasi-molecular ion $[M-H]^-$ of M5 was 153, which was identified to be PAC oxidized from PAL (M10). And M11 was determined as VAC (methylated conjugate of PAC) by comparing its retention time and UV absorption with those of VAC standard in HPLC chromatogram.

3.2.2. Glucuronided conjugates

Quasi-molecular ion $[M-H]^-$ for M1 and M3 was 329, and the product ions were 175, 113, 153, showing that M1 and M3 were the glucuronided conjugates of PAC, because 175 and 113 were the product ions of glucuronide [11,20] and 153 was the quasi-molecular ion $[M-H]^-$ of PAC. Quasi-molecular ion $[M-H]^-$ of M2 and M6 was 343, and the product ions were 175, 113, 167. The product ion at 167 was 14 mass unit higher than that of PAC. Therefore, M2 and M6 were supposed to be the glucuronided conjugates of methylated PAC. Quasi-molecular ion $[M-H]^-$ of M4 and M7 was 313, and the product ions were 175, 113, 137, showing that M4 and M7 were the glucuronided conjugates of PAL after oral administration of PAL to rats.

3.2.3. Glycine conjugates

The appearance of the predominant product ion at *m/z* 224 and a series of product ions at *m/z* 180 (224–44), 165 (224–59), 123 (224–101) and 100 in the MS^2 spectrum of the molecular ion of M8 and M9 showed that they were the glycine conjugates of methylated PAC because it is the cleavage feature of –COOH, –CH_2–COOH and –CO–NH–CH_2–COOH to lose 44, 59 and 101 Da, respectively.

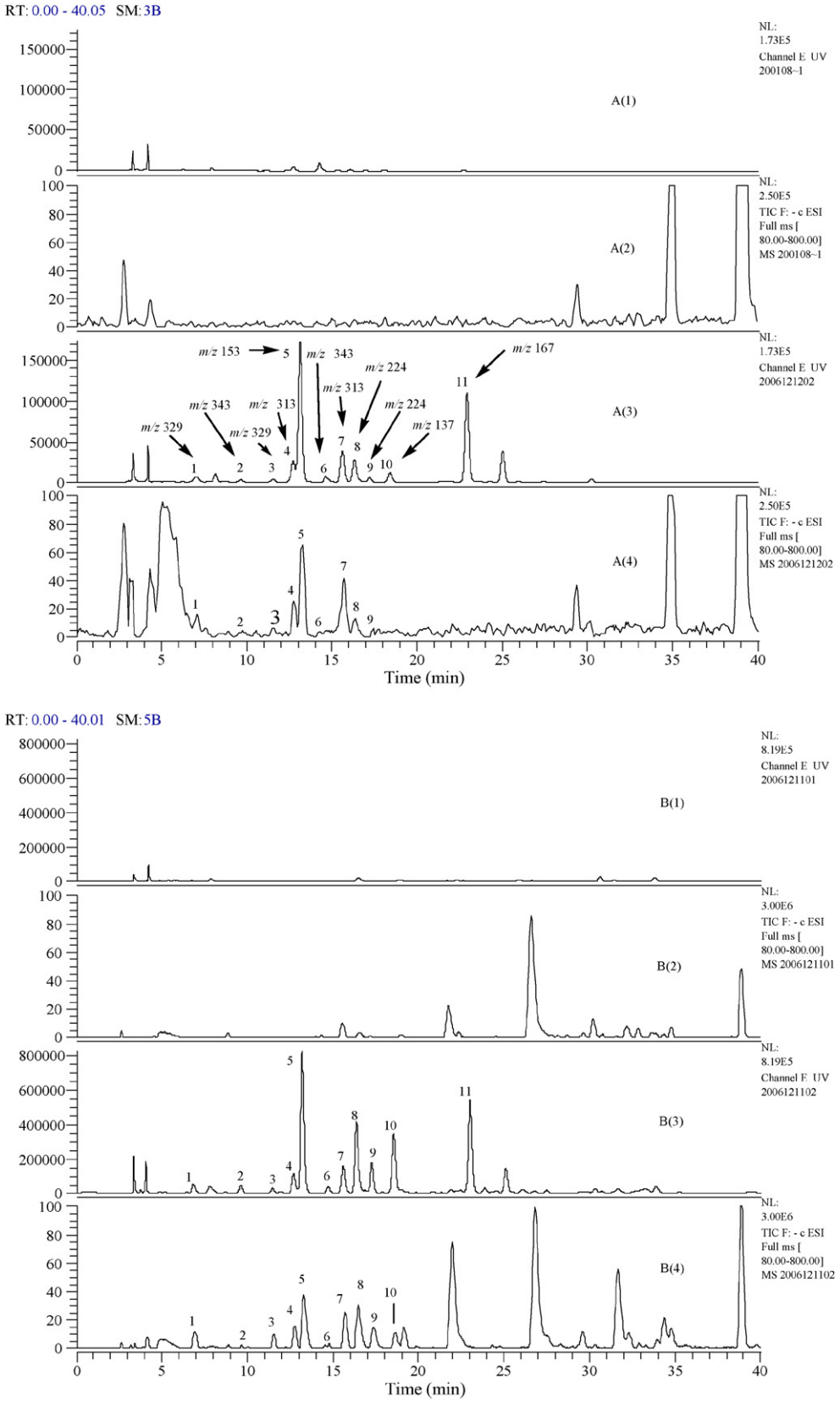

Fig. 1. UV (288 nm) and total ion current (TIC) chromatograms of blank samples and rat plasma, urine and bile samples after oral administration of PAL. (A) Rat 15 min plasma sample, (B) rat 0–10 h urine sample, (C) rat 0–10 h bile sample. (1) UV chromatograms of blank samples, (2) TIC chromatograms of blank samples, (3) UV chromatograms of dosed samples, (4) TIC chromatograms of dosed samples. Peak numbers on the profiles were identical to those shown in Table 1.

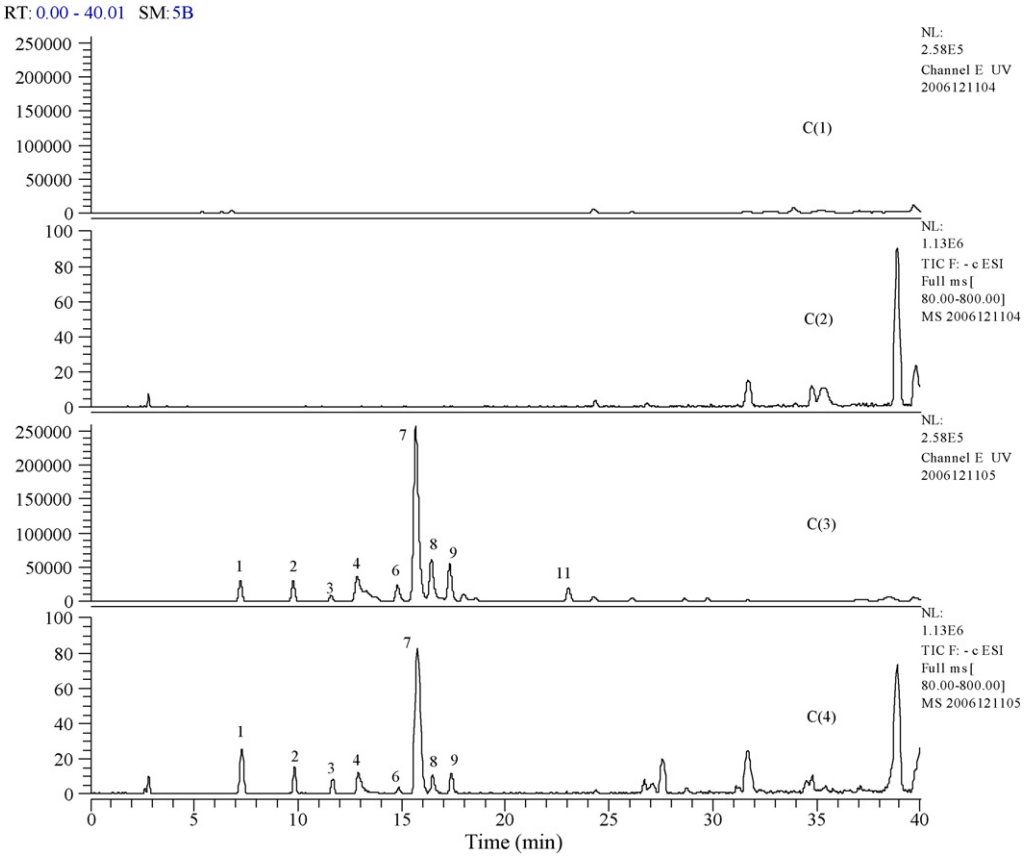

Fig. 1. (*Continued*).

By comparing the profiles of plasma, urine and bile samples after dosing of PAL, it was shown that 11 metabolites were detected in plasma and urine samples *in vivo*, but PAL and PAC were not detected in the bile sample. And the level of total PAL compounds (free and conjugate forms) reached a maximum concentration at 15 min after PAL administration by comparing the UV chromatograms of plasma samples from 5 min to 120 min (data not shown).

There is no obvious difference between the chromatogram of blank feces sample and that of 0–24 h feces sample after oral administration of PAL. This indicated that PAL and its metabolites should be excreted from the body via rat urine and bile, and enthrohepatic circulation should exist.

3.3. Elucidation of the possible metabolic pathway after oral administration of PAL

Biotransformation of drug proceeds in (at least) two distinct steps. During the 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) and the phase I biotransformation is a necessary prerequisite for the subsequent conjugation. In the second step (phase II), primary metabolites undergo conjugation reactions to form secondary metabolites. Phase II biotransformation leads not only to an inactivation of primary metabolites, but also to an increased hydrophilicity, hence secondary metabolites except acetylation and methylation showed enhanced excretion. Meanwhile, conjugation increases the molecular weight leading to more amenable in biliary excretion [22]. The purpose of this study was to examine the absorption, metabolism and excretion of orally administered PAL in a strain of experimental rats by using HPLC–MS^2 method.

All PAL metabolic products (free and conjugate forms) which could be detected at 5 min in the plasma reached the peak level at 15 min after PAL administration according to the HPLC-UV chromatograms of plasma samples from 5 min to 120 min. This result showed that PAL and its metabolites were rapidly absorbed from the digestive tract after oral administration of PAL. However, we cannot determine where the absorption of PAL and its metabolites occurred. The occurrences of PAL and its primary metabolite PAC in plasma showed that the aldehyde moiety in PAL might undergo oxidation to form the carboxyl moiety. Moreover, the majority of metabolites were present in the plasma as conjugated forms including glucuronided, methylated and glycine conjugates. A previous report showed that the activity of uridine-diphosphate-glucuronosyltransferase (UGT) in the liver was higher than that in the intestinal micosa [23] and thus rat intestine, together with liver, was regarded as the primary site of first-pass glucuronidation of PAL and PAC. Meanwhile, cathecol-*O*-methyltransferase (COMT) catalyzed the transfer of

Table 1
HPLC/MS^2 data and *in vivo* identification of metabolites in rat plasma, urine and bile

Peak no.	Retention time (min)	Assigned identify	Plasma metabolites	Urine metabolites	Bile metabolites	$[M-H]^-$ *m/z*	HPLC/ESI-MS^2 *m/z* (% base peak)
1	6.85	Protocatechuic acid monoglucuronide	+	+	+	329	MS^2[329]: 175 (60), 153 (100), 113 (45)
2	9.61	Methylated protocatechuic acid monoglucuronide	+	+	+	343	MS^2[343]: 175 (100), 167 (20), 113 (78)
3	11.47	Protocatechuic acid monoglucuronide	+	+	+	329	MS^2[329]: 175 (40), 153 (100), 113 (45)
4	12.69	Protocatechuic aldehyde monoglucuronide	+	+	+	313	MS^2[313]: 175 (21), 137 (100), 113 (17)
5	13.19	Protocatechuic acid*	+	+	–	153	MS^2[153]: 109 (100)
6	14.72	Methylated protocatechuic acid monoglucuronide	+	+	+	343	MS^2[343]: 175 (100), 167 (25), 113 (80)
7	15.61	Protocatechuic aldehyde monoglucuronide	+	+	+	313	MS^2[313]: 175 (16), 137 (100), 113 (20)
8	16.37	Methylated protocatechuic acid monoglycine	+	+	+	224	MS^2[224]: 180 (5), 123 (20), 100 (100)
9	17.27	Methylated protocatechuic acid monoglycine	+	+	+	224	MS^2[224]: 180 (55), 165 (100), 100 (5)
10	18.55	Protocatechuic aldehyde*	+	+	–	137	–
11	23.02	Vanillic acid*	+	+	+	–	–

* Positively identified via comparison with authentic standards, see Fig. 2.

the methyl group from SAM to the hydroxyl group of phenolic compounds with a catechol structure and the hepatic enzyme may mainly contribute to the *in vivo O*-methylation of phenolic acids [24,25]. It was also found that glycine conjugates were present in the plasma. According to the literature [26,27], glycine conjugation depends not only upon medium chain acyl-CoA synthetases but also upon acyl-CoA: glycine *N*-acyltransferases in the liver and kidney. And glycine *N*-acyltransferase which transfers benzoyl groups from acyl CoA compounds to glycine has been isolated from beef liver mitochondria [28]. For example, 3-hydroxybenzoic acid was conjugated with glycine in the liver and kidney to form 3-hydroxuhippuric acid [29]. Piskula and Terao reported that when (−)-epicatechin is absorbed from the digestive tract, it was conjugated with glucuronic acid by UGT and the conjugated form is methylated by COMT in the liver and kidney [30]. In the present study, several similar metabolites such as glucuronided, methylated, glycine, methyl-glucuronided and methyl-glycine forms of PAL and PAC were detected in the plasma. Thus, according to the above-mentioned literatures, we might speculate that the glucuronided conjugates of PAC would occur in the rat plasma followed by methylation in the liver. There are two potential hydroxyl moieties in PAL and PAC for conjugating, which indicated that further studies should be focused on the clarification of conjugating sites in PAL and PAC. The current HPLC–MS^2 method fails to precisely identify the structures of phenolic acids in the form of monomer. One possible pathway proposed for PAL metabolism following oral administration to rats is shown in Fig. 2.

Previous studies in rats investigating polyphenolic substances such as phenolic acids, flavonoids and curcuminoids have shown that the majority of the metabolites of these compounds were present in rat plasma, urine or bile as conjugates including methylated, glucuronided and sulfated conjugates [23,25,31–34]. Our study demonstrated that orally administered PAL was oxidized to PAC in the phase I metabolism and occurred in the plasma in the form of PAL, PAC and their conjugates, most of which were excreted in rat urine and bile within 10 h after administration. The difference between urinary and biliary excretion lies in the presence of PAL and PAC in rat urine. It was illustrated that PAL and PAC should be excreted from the body via rat urine. It was reported that deconjugation of rosmarinic acid metabolites might occur in the renal pathway catalyzed by β-glucuronidase located in the kidney [31,32]. Hence, PAL and its primary metabolite PAC which were detected in the urine may be from their prototypes in the plasma and their glucuronided conjugates deconjugated in rat kidney. On the other hand, biliary excretion was also found in this study with the excretion of conjugates of PAL and PAC. A previous study showed that β-glucuronidase occurred highly in enterobacteria and clostridia, and bisphenol A-glucuronide was rapidly excreted into the small intestine via the bile duct and deconjugated in the cecum of the rat [35]. Therefore, a part of glucuronided conjugates of PAL and PAC excreted into bile may be converted to PAL and PAC and reabsorbed. This confirms the proposition by Kuchimanchi et al. that molecules with a large molecular weight are easily excreted into the bile, and the threshold molecular weight of biliary excretion should be 325 ± 50 in rats [36]. The reason why

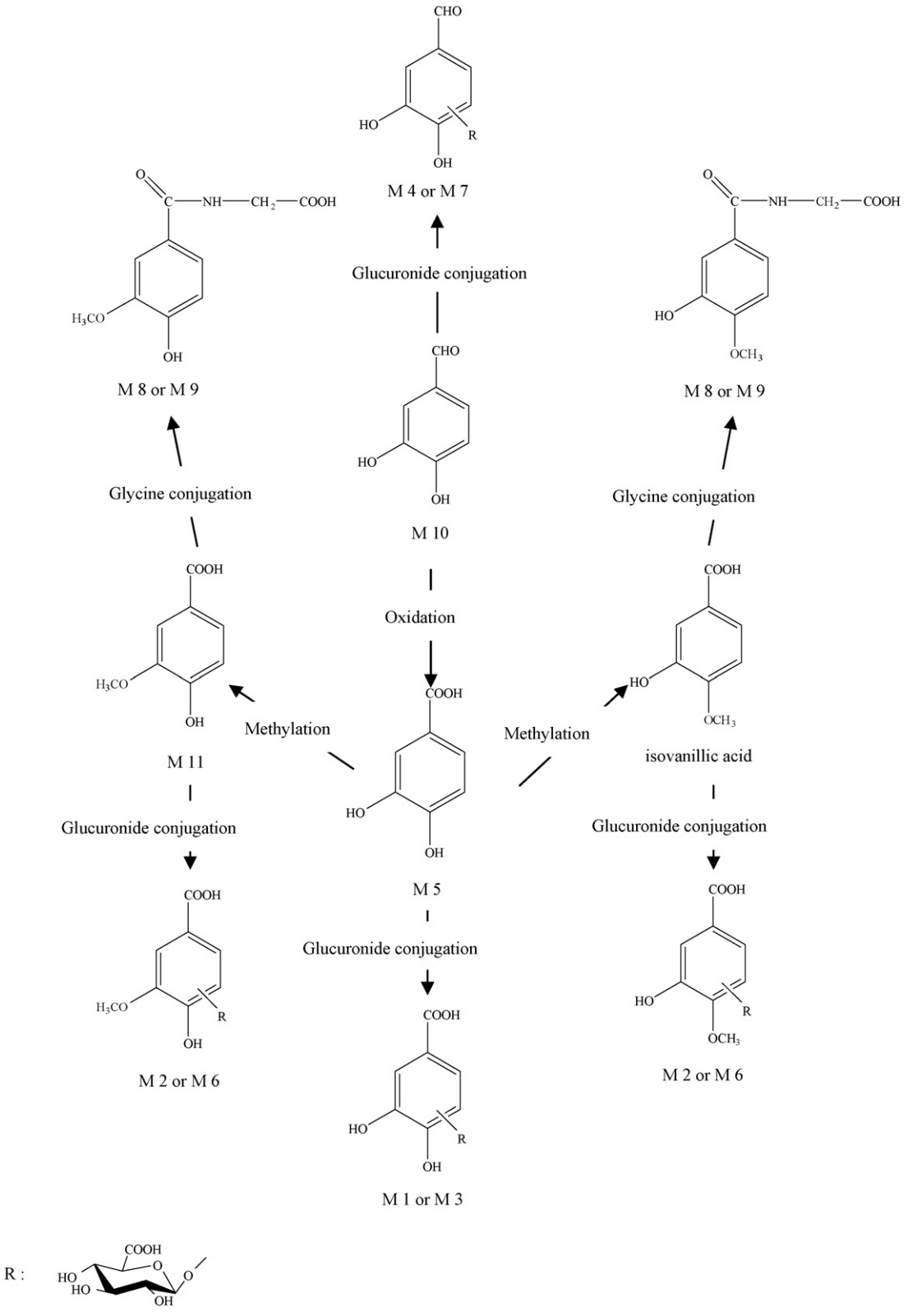

Fig. 2. Proposed scheme of possible metabolic pathway of PAL administered orally in rats.

PAL and PAC (molecular weights were 138 and 154, respectively) could not be excreted from the bile was also illustrated. Meanwhile, the evidence that no metabolites occurred in the feces indicated that enthrohepatic circulation might exist after PAL administration, which could prolong the presence of PAL and its metabolites in the systemic circulation.

Ross and Wootton reported that all the benzoic acids with a free phenolic group showed a marked degree of inhibiting

enzymes which were important to the cerebral metabolism, and those with dihydroxy groups had the greatest effect. On the other hand, methylated phenolic groups had no effect, while glycine conjugates, in general, stimulated the activities of enzymes aforementioned [28]. As to PAL, it is important to elucidate whether the metabolites of PAL lead to inactivation (detoxification) or activation (bioactivation).

4. Conclusions

An HPLC–ESI-MS/MS method was developed for the identification of rat metabolites *in vivo* in plasma, urine and bile after oral administration of PAL. For the first time, 11 metabolites were inferred to be methylated, glucuronized and glycine conjugates of PAL and PAC. The proposed method was simple, reliable and sensitive, revealing that it was appropriate for rapid screening and structural characterization of rat metabolites *in vivo* after dosing of PAL. Furthermore, the possible metabolic pathway of PAL was proposed. PAL should be oxidized to PAC at first. And the conjugates would occur in rat intestine, liver and kidney and excreted in rat urine and bile. Enthrohepatic circulation was also found in this study. This investigation provided scientific evidence to infer the genuine active components responsible for the pharmacological effects of PAL. It was also helpful to better understand the *in vivo* metabolism of PAL. Further studies are required to identify which of the PAL metabolites are active in inducing a variety of beneficial physiological functions in animals and humans.

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