Investigation of piceid metabolites in rat by liquid chromatography tandem mass spectrometry

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ABSTRACT

There is considerable evidence that stilbenes provide health benefits. *Trans*-piceid is one of the major stilbenoid compounds in red wine and other plants. The purpose of this study is to investigate the metabolism of piceid in rats, including its conversion product by intestinal microflora in vitro and urinary metabolites. A HPLC–MS/MS method with electrospray ionization (ESI), negative ion mode and collision induced dissociation (CID), was used to elucidate the structures of the major metabolites of piceid. Three metabolites resveratrol, dihydropiceid and dihydroresveratrol were detected after incubating with gut microbiota for 5 h. Four urinary metabolites of piceid were identified as resveratrol, dihydroresveratrol monosulfate, piceid monosulfate and piceid monoglucuronide.

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1. Introduction

Piceid (3,4′,5-trihydroxystilbene-3-β-mono-D-glucoside) is a glucoside of the stilbene-like resveratrol. These are phenolic compounds present in many families of plants such as grapes, *Polygonum cuspidatum*, peanuts, and so on. Wine is considered the most important dietary sources of these substances [1].

Piceid and resveratrol occur as *trans*- and *cis*-isomers. The chemical structure of *trans*-piceid is shown in Fig. 1. Due to the generally high ratio of *trans*- to *cis*-isomers found in wines and other plants, it has been suggested that the *cis*-isomers could arise from light exposure [2].

Piceid has received such attention as resveratrol because, in many plants and related products, the concentration of the glucoside is usually significantly higher than the aglycone [2,3]. Piceid isomers have properties similar to those of resveratrol in inhibiting platelet aggregation [4–6] and inhibiting oxidation of human low-density lipoproteins. On the other hand, in a less active manner than *trans*-resveratrol, *trans*-piceid reduces the elevations of lipid levels and inhibits eicosanoid synthesis [7].

Although many studies have implicated a role of piceid in disease prevention, only a few studies have addressed its bioavailability and metabolism. In vitro studies indicated that *trans*-piceid was deglycosylated in Caco-2 cells, and the resulting aglycone was metabolized into *trans*-resveratrol-3-O-β-glucuronide and to a lesser extent into *trans*-resveratrol-4-O-β-glucuronide. When *trans*-piceid was incubated with Caco-2 cells, *trans*-resveratrol was detected on both apical and basolateral sides [8]. In vivo studies have shown that *trans*-piceid, *trans*-resveratrol, *trans*-resveratrol-3-O-β-glucuronide, glucuronidated *trans*-piceid and sulfated *trans*-piceid are detectable in plasma after oral administration of *trans*-piceid to men and rats [9–11].

In this paper, we investigated the specific degradation products of piceid by gut microflora, and identified the metabolites excreted in rat urine after oral administration of piceid. To our knowledge, three metabolites of piceid dihydropiceid, dihydroresveratrol and dihydroresveratrol monosulfate were the first reported in our experiments.

2. Experimental

2.1. Chemicals and reagents

Trans-piceid (>99%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products. HPLC-grade methanol was purchased from Merk KGaA (Darmstadt, Germany). Water was of deionized distilled water.

2.2. Animals

Six male Sprague-Dawley rats weighing 200 ± 30 g were obtained from the Breeding Laboratories in nantong university. Rats were housed in metabolic cages with water and a solid diet freely available, and maintained at 22 ± 3 °C with 40–70% relative humid-

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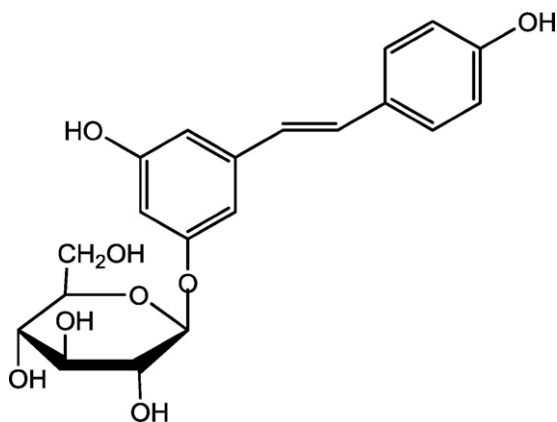 E-mail address: wdg1228@126.com (D. Wang).

Fig. 1. Chemical structures of *trans*-piceid.

ity. Rats were acclimatized under these conditions for at least 2 days before doing our experiments. The research using rats adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985).

2.3. In vitro fermentation experiment

Three rats were sacrificed by decapitation. Small and large intestines were excised, and their contents were diluted at a ratio of 1:3 (v/v) with physiological saline solution. Fermentation bottles with piceid (1.5 mg) was inoculated with diluted contents (10 ml) and incubated with stirring in anaerobic conditions at 37 °C. Content without being added peceid compound was used as a control. After incubation, the conversion was stopped by adding methanol to samples at a ratio of (2:1). The mixture was immediately filtered, and then the solution was concentrated and filtered for HPLC and HPLC–MS/MS detection.

2.4. Collection and pretreatment of rat urine sample

A dose of 20 mg/kg of piceid dissolved in a hydro-alcoholic solution (approx. 3.0%, v/v ethanol in water) was orally administered by gastric intubation to overnight fasted rats. Urine samples were collected from rat at −12 to 0 h predose and at 12 h postdose, and stored at approximately −20 °C before the sample preparation.

Urine samples were thawed in a water bath at room temperature and then centrifuged at approximately 3000 × g for 5 min. Polyamide SPE cartridges (180 mg) were conditioned with 5 ml of methanol and 10 ml of water. Aliquots of 1 ml urine samples were loaded onto the cartridges, which were washed with 10 ml of water and eluted with 4 ml of methanol. The organic layer was evaporated to dryness under nitrogen at 40 °C. The residues were dissolved in 200 μl of 100% methanol for HPLC and HPLC–MS/MS detection.

2.5. HPLC analysis

The analysis was carried out on a HPLC system (Shimadzu, Japan) equipped with a LC-20AD pump, SPD-20A detector, AT-300 column oven. A Nucleosil 100 C18 reverse-phase column (150 mm × 4.6 mm; particle size, 5 μm; Knauer, Berlin, Germany) protected by a pre-column was used.

For determination of samples obtained in this experiment, we used acetic acid–methanol–water (2:10:90, v/v) as solvent A, and 100% methanol as solvent B, at a flow rate of 1.0 ml/min with the following gradient: 0–50% B linear (0–15 min), 50–100% B linear (15–18 min), 100% B (18–23 min). This was followed by a 15 min equilibrium period with initial conditions prior to injection of the next sample. Samples were filtered (0.45 μm, Millipore) and 20 μl was directly injected. Chromatograms were monitored at 290 nm using the UV detector.

2.6. HPLC/MS/MS analysis

HPLC/MS/MS analyses were performed using a system consisting of a Finnigan autosampler (Thermo Electron Corperation, USA), a Finnigan LC pump, a Finnigan TSQ Quantum Ultra equipped with an electrospray ion source and operated by XCalibur software.
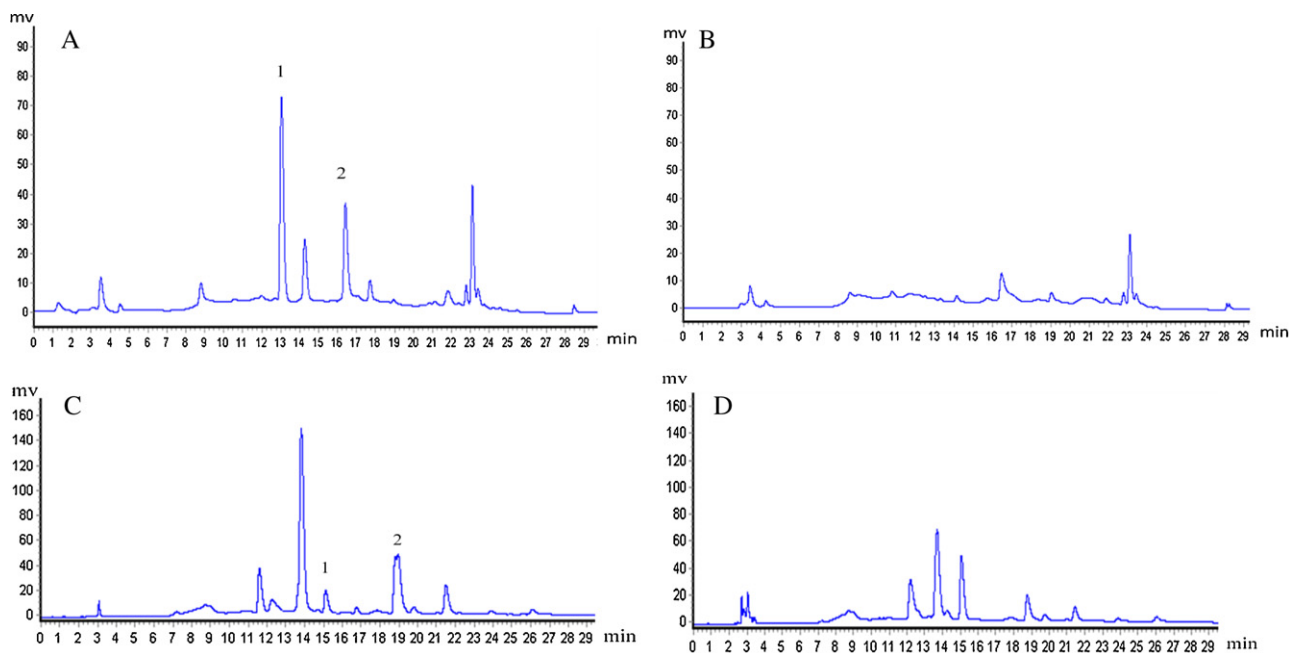

Fig. 2. Representative HPLC chromatograms for intestinal incubated products (A and B) and rat urine samples (B and C). (A) chromatogram of sample incubated for 5 h, (B) blank, (C) rat urine sample collected during 0–7 h, and (D) blank urine.
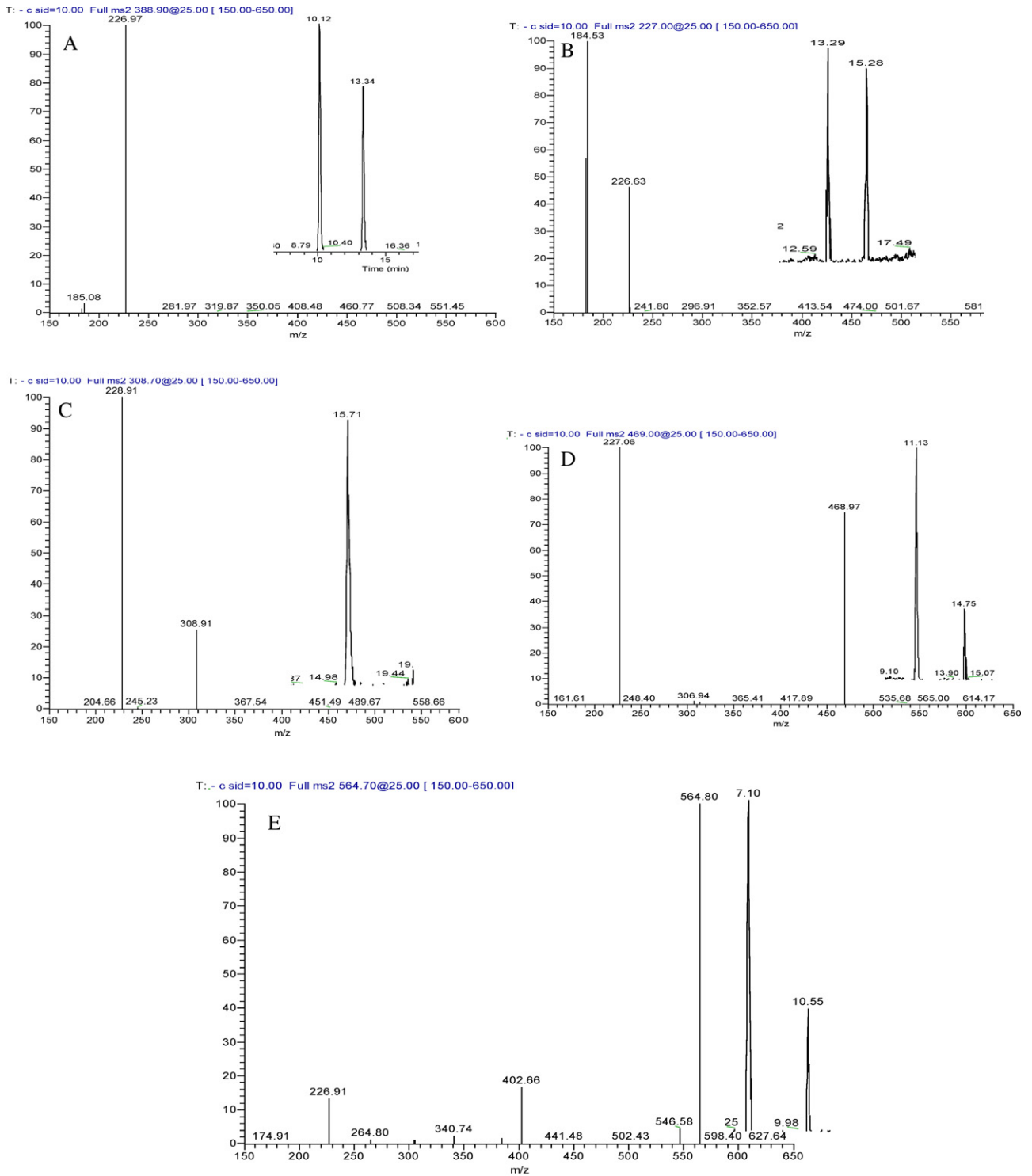
Fig. 3. HPLC–MS/MS spectra of piceid and its metabolites obtained from rat urine after oral administration of piceid (20 mg/kg). (A) piceid M0, (B) resveratrol M1, (C) dihydroresveratrol monosulfate M2, (D) piceid monosulfate M3, and (E) piceid monoglucuronide M4.

The separation was carried out by using a C_{18} reverse-phase column (100 mm × 3 mm; particle size, 5 μm; Shimadzu, Japan) protected by a pre-column. We used 10% methanol as solvent A, and 100% methanol as solvent B, at a flow rate of 1.0 ml/min with the same gradient condition as that mentioned above.

The mass spectral analysis was performed in a negative electrospray ionization mode. The capillary and orifice voltages were set at −3.7 kv and −65 v, respectively. The nebulizer gas was set at 50 psi. The nitrogen auxiliary was adjusted to a constant flow rate of 2 l/min. The capillary temperature was set at 400 °C. Collision induced dissociation (CID) studies were performed using collision energy of 30 eV. The ion spray interface and mass spectrometric parameters were optimized to obtain maximum sensitivity at unit resolution.
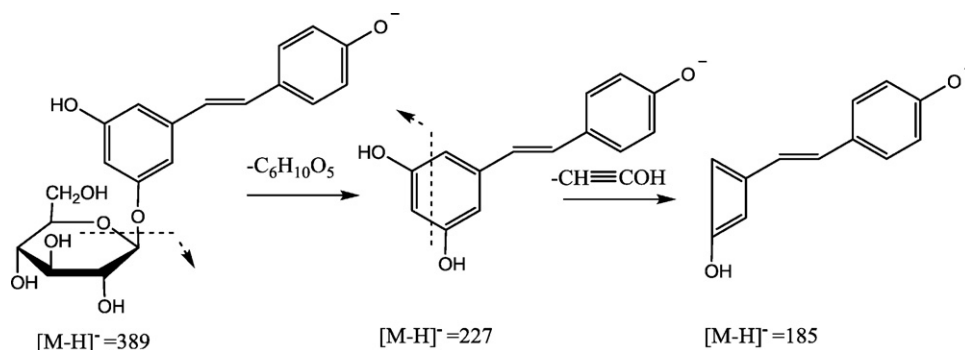
Fig. 4. Proposed mechanism for the decomposition of the m/z 389 [M−H]$^-$ ion of piceid (M0).
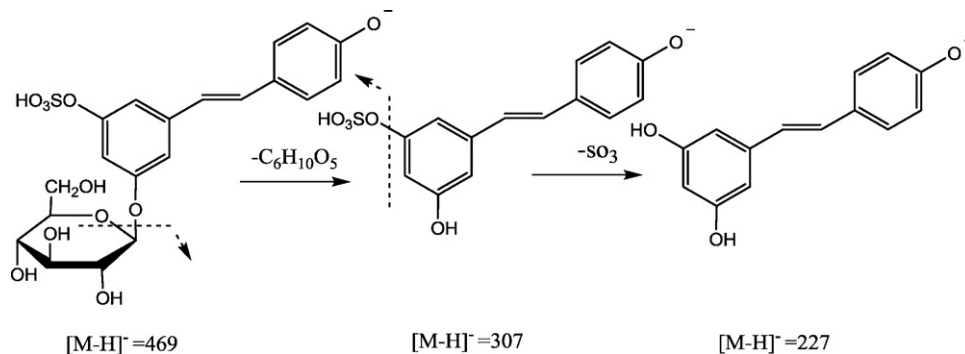

Fig. 5. Proposed mechanism for the decomposition of the m/z 469 [M−H]$^-$ ion of piceid monosulfate (M3).

3. Results and discussion

3.1. HPLC chromatographs

After being incubated with gut microbiota, the metabolites of piceid were determined by HPLC, and chromatograms of these samples were illustrated in Fig. 2A and B. By comparison with the blank and reference solution, It was clear that peak1and 2 with retention time of 13.0 and 16.5 min were of piceid and resveratrol respectively, while others were of unknown components.

In order to eliminate the endogenesis interferer, rat urine samples were cleaned up by using solid-phase extraction (SPE) with polyamide cartridges. The chromatograms of these samples were illustrated in Fig. 2C and D. In addition to piceid (peak1) and resveratrol (peak2), we can also find other peaks of unknown metabolites.

3.2. Microbial metabolism of piceid

After being incubated with gut microbiota, the metabolites of piceid were analyzed by HPLC–ESI–MS–MS, based on the respective m/z values of parent and product ions (Table 1). Three metabolites

Table 1
Peceid metabolites looked for in the gut fermentation samples by tandem mass spectrometry. Negative ionization was used.

Compounds	Molecular weight	Parent ion (m/z)	Main Product ion (m/z)
Parent compound			
Peceid	390	389	227
Metabolites			
Resveratrol	228	227	185
Dihydropiceid	392	391	229
Dihydroresveratrol	300	299	187

were identified as resveratrol, dihydropiceid and dihydroresveratrol.

Among these metabolites, resveratrol was the major microbial degradation product formed by deglycosylation, while dihydropiceid and dihydroresveratrol were formed by reduction of a double bond. This is consistent with those results of compounds with similar structure [12].

3.3. Identification of the metabolites of piceid in rat urine

3.3.1. piceid (M0)

As illustrated in Fig. 3A, the negative electrospray mass spectrum of piceid (*trans*-piceid Tr = 10.1 min, *cis*-piceid Tr = 13.3 min) showed a [M−H]$^-$ ion at m/z 389. The CID spectrum of m/z 389 generated fragment ions at 227 and 185. The fragment ion at m/z 227 was generated by deglycosylation from the loss of 162($C_6H_{10}O_5$). The m/z 227 further fragmented to m/z 185 after loss of 42(C_2H_2O). The fragmentation pathway may be explained according to Fig. 4.

3.3.2. Metabolite M1

The mass spectrum of M1 was listed in Fig. 3B. The negative electrospray mass spectrum of M1 (Tr = 13.3 min) showed a [M−H]$^-$ ion at m/z 227. The CID spectrum of m/z 227 generated fragment ion at 185, which was generated after the loss of 42(C_2H_2O). This result is consistent with that of our previous report [13], so it can be identified as resveratrol. For the possible decomposition mechanism of these fragment ions refers to our previous report [13].

3.3.3. Metabolite M2

The mass spectra of M2 were listed in Fig. 3C. The [M−H]$^-$ ion of M2 (Tr = 15.7 min) was at m/z 309. The m/z 309 was 2 Da higher than that of resveratrol monosulfate, and was in agreement with that of dihydroresveratrol monosulfate. The CID spectrum of M2 yielded fragment ions at m/z 229. The m/z 229 was 2 Da higher than that of resveratrol (m/z 227), and was consistent with dihydroresvera-
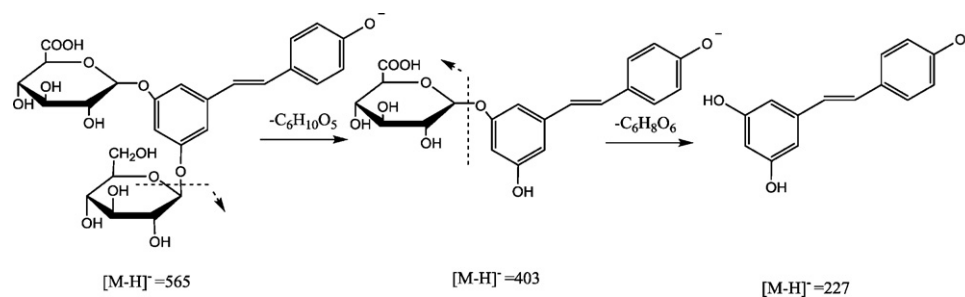
Fig. 6. Proposed mechanism for the decomposition of the m/z 565 [M−H]$^-$ ion of piceid monoglucuronide (M4).
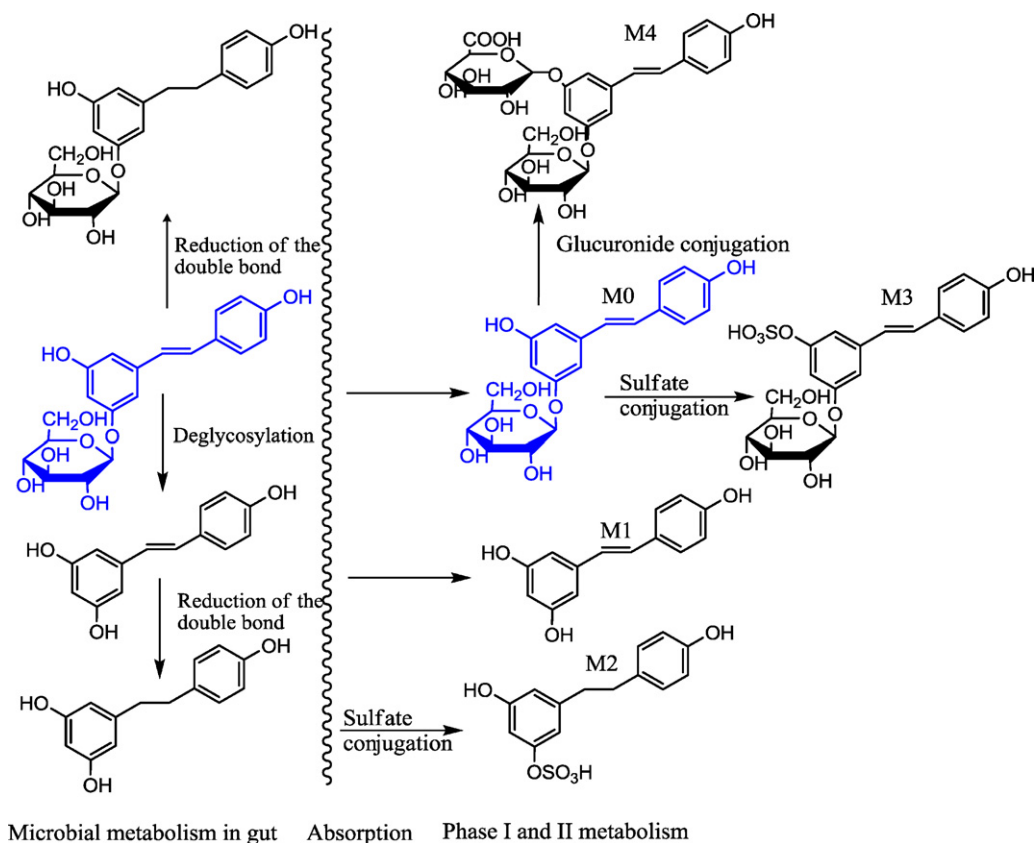

Fig. 7. The proposed metabolic pathway of piceid in rat.

trol. The m/z 229 was formed from m/z 309 after loss of 80(SO$_3$). Therefore, M2 can be identified as dihydroresveratrol monosulfate. For the possible decomposition mechanism of these product ions refers to our report [13].

3.3.4. Metabolite M3

The [M−H]$^-$ ion of m/z 469 (M3, see Fig. 3D (Tr = 11.1) fragmented to form a product ion of m/z 227 that corresponded to resveratrol after the loss of sulfate and C$_6$H$_{10}$O$_5$. Therefore, the peak at 11.1 min was identified as piceid monosulfate (MW = 470). Only one piceid monosulfate and no disulfate metabolites were detected. The fragmentation pathway may be explained in terms of Fig. 5.

3.3.5. Metabolite M4

The mass spectra of M4 were listed in Fig. 3E. Full mass of M4 which was detected at 7.1 min gave a [M−H]$^-$ ion at m/z 565, 176 Da higher than that of piceid. The CID spectrum of M4 showed fragment ions at m/z 403, 227. m/z 403, which was corresponded to

resveratrol glucuronide, was formed after the loss of C$_6$H$_{10}$O$_5$. The ion at m/z 227 was formed after the loss of a glucuronic acid moiety and C$_6$H$_{10}$O$_5$. Therefore, the peak at 7.1 min was identified as piceid monoglucuronide. The fragmentation pathway may be explained according to Fig. 6.

According to our experimental results including the conversion product by intestinal microflora and urinary metabolites, the metabolic pathway of piceid was proposed as shown in Fig. 7.

So far, there have been several reports on piceid metabolism in vivo and in vitro. It is reported that the metabolites of piceid were resveratrol, glucuronidated resveratrol, glucuronidated piceid and sulfated resveratrol. Our study indicates that after being incubated with gut microbiota, piceid can be transformed to resveratrol, dihydropiceid and dihydroresveratrol, and that metabolites of resveratrol, dihydroresveratrol monosulfate, piceid monosulfate and piceid monoglucuronide could be found in rat urine after oral administration of piceid. To our knowledge, three metabolites of dihydropiceid, dihydroresveratrol and dihydroresveratrol monosulfate were first reported in our experiments.

4. Conclusions

As a glycosylated polyphenol, piceid could be transformed to aglycone by β-glucosidases from bacteria and/or epithelial cells in gut with the hydrolysis of glycosidic unit. Also there is a aliphatic double bond in its molecular structure, so reduction reaction occurs in the presence of intestinal microflora. After that these microbial metabolites can be absorbed and metabolized in the liver by phase II enzymes as conjugated metabolites, and finally they are excreted in faeces and urine. Therefore, the urinary metabolites resveratrol, dihydroresveratrol monosulfate, resveratrol glucuronide might arise from the intestinal microbial metabolites.

Acknowledgements

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