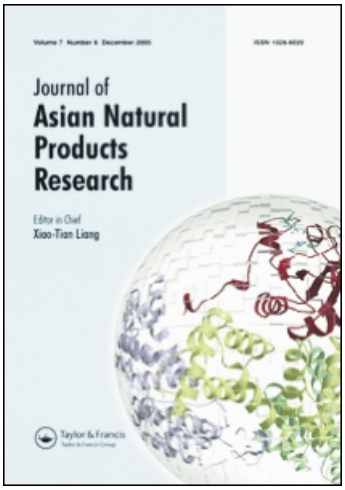

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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To cite this Article Zhang, Jin-Lan , Zhang, Guo-Dong and Zhou, Tong-Hui(2005) 'Metabolism of ferulic acid in rats', Journal of Asian Natural Products Research, 7: 1, 49 — 58
To link to this Article: DOI: 10.1080/10286020310001617129
URL: http://dx.doi.org/10.1080/10286020310001617129

Journal of Asian Natural Products Research, Vol. 7, No. 1, February 2005, 49–58

Metabolism of ferulic acid in rats

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(Received 25 March 2003; revised 18 June 2003; in final form 28 June 2003)

Ferulic acid is the major active constituent in many natural Chinese medicinal herbs. The metabolism of ferulic acid has been investigated using solid-phase extraction and HPLC-DAD methods that were established to separate and analyze the metabolites in urine, feces and bile. Three metabolites, identified by enzymatic hydrolysis, HPLC-DAD, HPLC-MS and MS/MS, are all glucuronic acid conjugates of ferulic acid. Ferulic acid conjugated with one glucuronic acid at different positions produces M1 and M3. Ferulic acid conjugated with two glucuronic acids produces M2, which is the main metabolite. A metabolic pathway is proposed.

Keywords: Ferulic acid; Metabolism; Metabolites; Wistar rats

1. Introduction

Ferulic acid is widely distributed in natural Chinese medicinal herbs, e.g. in the rhizoma and roots of *Ligusticum chuanxiong Hort, Ligusticum jeholense Naika et Kitagawa, Angelicae sinensis, Cimicifuga foetida L.*, and *Cimicifuga heracleifolia*. Ferulic acid has obvious pharmacological activity as anti-inflammatory [1,2], sedation [3], anti-oxidation [4,5], protection against beta-amyloid peptide toxicity *in vivo* [6] and improving viability and motility in both fertile and infertile individuals [7,8]. It is clinically used to cure cerebral thrombosis, hemicrania and cerebrovascular diseases caused by ischemia. Studies of ferulic acid have mainly touched upon its pharmacological activity [1–8] and pharmacokinetics in rabbits, rats and dogs [9–12]. Here we studied the metabolism of ferulic acid in rats. Three metabolites were conjugated compounds of ferulic acid with β-glucuronic acid and were identified by enzymatic hydrolysis, HPLC-DAD and HPLC-MS. They have not been reported before in natural plants.

2. Results and discussion

2.1 Sample preparation and HPLC-DAD analysis

Urine obtained during 0–12 h after oral administration of ferulic acid was used to select favorable extraction conditions and the HPLC-DAD analytical method. The urine was

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Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2005 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020310001617129

adjusted to pH 3.5 by 10% HCl, then the same volume of acidified urine was extracted by EtOAc and ODS separately. In the meantime the same volume of non-acidified urine was directly subjected to ODS extraction. The results showed that the metabolites and parent drug were extracted at a relatively high rate when the acidified urine was subjected to ODS, but only the parent drug was extracted at a high rate when acidified urine was extracted by EtOAc. When the urine was directly subjected to ODS without acidification, the metabolites were concentrated and the parent drug was extracted at a low rate. Therefore, the extraction of acidified urine with ODS was used to evaluate the metabolism and excretion rate of ferulic acid, and the direct extraction with ODS of urine without acidification was used for metabolite identification by HPLC-MS (figure 1).

Urine, feces and bile during 0–12 h and 12–24 h after oral administration of ferulic acid to rats were analyzed by the established HPLC-DAD method. The HPLC-DAD chromatograms (figure 2) show three metabolites in the urine, by comparison with blank samples and the UV spectrum of the parent drug. The metabolites have a similar UV profile to that of ferulic acid, and are designated as M1, M2 and M3 in order of decreasing polarity. The results show that ferulic acid was metabolized in the rats at a low rate and mainly excreted from urine in the form of the parent drug. There were small amounts of parent drug in feces, and nothing in the bile.

2.2 Enzymatic hydrolysis

Urine obtained during 0–12 h after oral administration of ferulic acid to rats was hydrolyzed by β-glucuronidase at 37°C for 12 h. HPLC chromatograms of hydrolyzed sample and unhydrolyzed samples were compared (figure 3). The M1 peak disappeared and M2 nearly disappeared, while M3 and the parent drug increased. We thus inferred that M2 and M1 were conjugates of M3 and the parent drug.

2.3 Metabolite identification by HPLC-MS and MS/MS

To obtain further structure information of the metabolites, urine samples collected during 0–12 h after oral administration were directly subjected to ODS extraction and analyzed by HPLC-MS. Both negative and positive mode ESI were performed, and showed that ESI in positive mode was sensitive to the metabolites. Molecular ions $[M + 1]^+$ of the three metabolites were collected (table 1 and figure 4). The molecular ions $[M + 1]^+$ of both M1 and M3 were 371, indicating the addition of a glucuronic acid (*m/z* 176) to ferulic acid (*m/z* 194). We presumed that glucuronic acid is conjugated with the 4-hydroxy group of ferulic acid to form M1. Glucuronic acid conjugated with the carboxyl group of ferulic acid forms M3. M1's polarity was higher than that of M3 because of the retention of the free carboxyl group. The molecular ion $[M + NH_4]^+$ of M2 was 564 and that of $[M + 1]^+$ was 547. We thus proposed that two glucuronic acids conjugate with ferulic acid to produce M2. The results of enzymatic hydrolysis further confirmed the above conclusion. M2 easily lost the glucuronic acid conjugated with the 4-hydroxy group to form M3 when hydrolyzed by glucuronidase. To confirm the structure of M2 it was prepared by preparative HPLC and analyzed by ESI^+-MS. Molecular ions $[M + NH_4]^+$ and $[M + 1]^+$ of M2 were observed. The molecular ion $[M + NH_4]^+$ was selected for further ionization and gave $[M + 1]^+$ of M2 and fragment 177 of glucuronic group in the MS/MS spectrum (figure 5). The results proved that M2 was a conjugate of ferulic acid with two glucuronic acids. The metabolite structures are shown in figure 6.

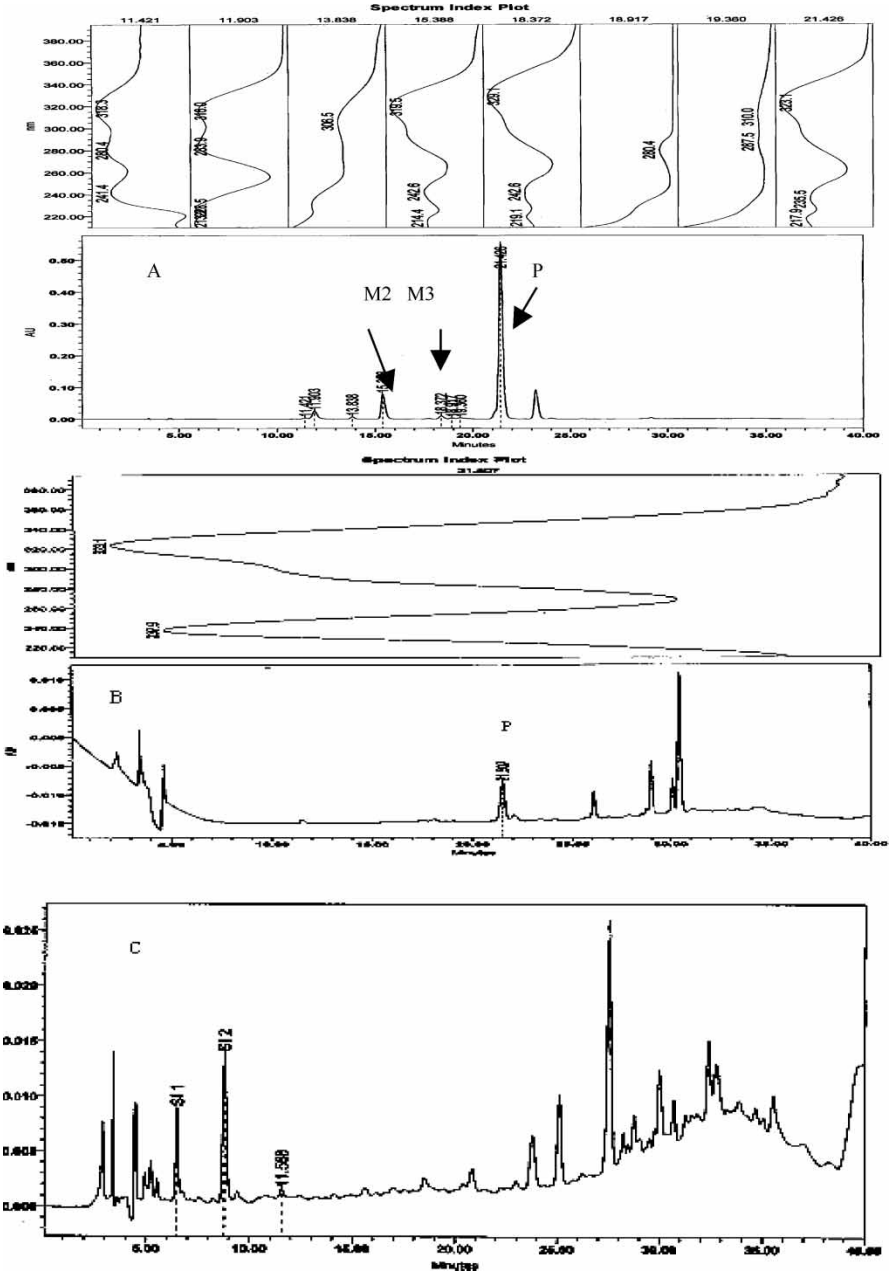

Figure 1. HPLC chromatogram of biological samples collected 0–12 h after oral administration of ferulic acid. (A): Urine, (B): feces, (C): bile. M2, M3: metabolites; P: ferulic acid.

2.4 Distribution of ferulic acid in rats

Two hours after oral administration of ferulic acid, the rats were decapitated and their visceras were excised and homogenized. The mixture was then centrifuged and the resultant upper clear solution was extracted with EtOAc after the pH had been adjusted to 3.5. The extracted samples were analyzed by HPLC. The results showed that ferulic acid had been distributed in the liver, kidney, spleen and lung, but it was not found in the heart after

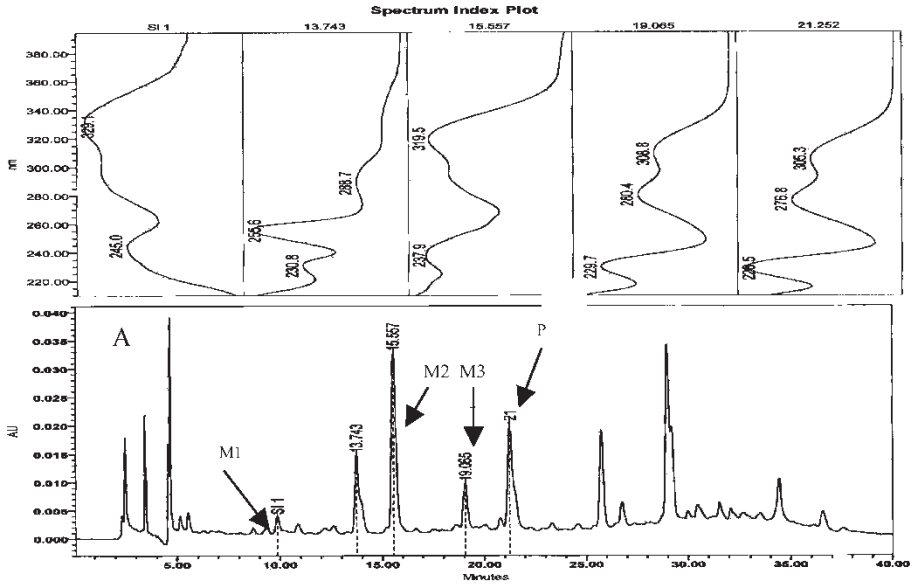

Figure 2. HPLC chromatograms of urine collected during 0–12 h after oral administration of ferulic acid (urine directly extracted by ODS without acidification): M1, M2, M3: metabolites; P: ferulic acid.

a two-hour oral administration (figure 7). A lot of ferulic acid remained in the stomach. There are many references on the pharmacokinetics of ferulic acid in serum after administrating ferulic acid or formulae [9–12], while the distribution of ferulic acid in an animal's organ and tissue has been little studied. Our study showed that ferulic acid was distributed widely in the rat's organ after oral administration and provided a method for further study of ferulic acid distribution in animals.

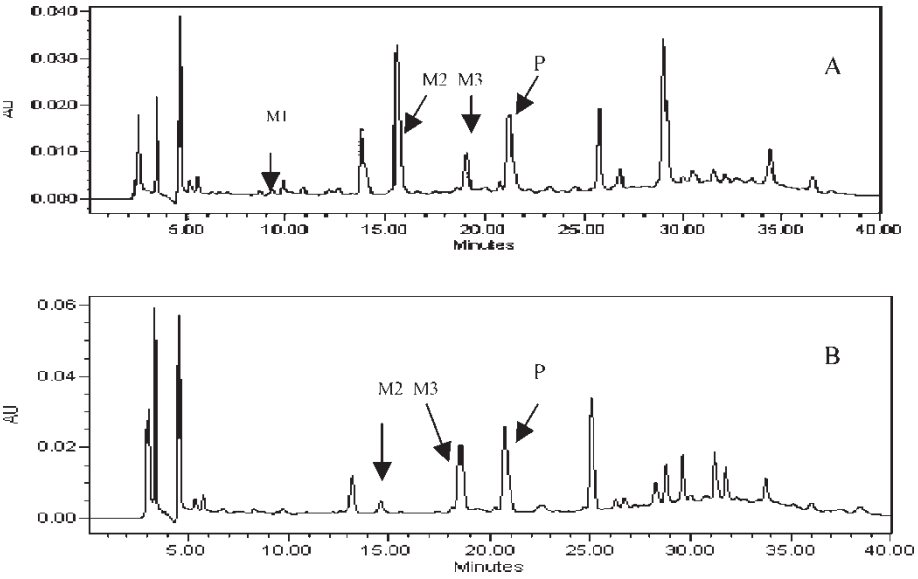

Figure 3. HPLC chromatogram of urine collected during 0–12 h and hydrolysis by β-glucuronidase. (A): Before hydrolysis, (B) hydrolysis by β-glucuronidase. M1, M2, M3: metabolites; P: ferulic acid.

Table 1. HPLC-MS data for parent drug and metabolites.

					ESI^+ data		
Metabolite	*Retention time (min)*		*UV λ (nm)*		$[M + M]^+$	$[M + NH_4]^+$	$[M + 1]^+$
M1	7.27	245.0	288.0	329.1		388	371
M2	14.7	237.9	288.4	319.5		564	547
M3	21.50	229.7	280.0	308.8	740		371

2.5 Pathway of metabolism and excretion

Ferulic acid was metabolized in rats at a low rate and mainly excreted from urine in the form of the parent drug after oral administration. There were small amounts of parent drug in the feces and nothing in biles. Three metabolites were detected. A possible pathway of metabolism is that ferulic acid is absorbed into the blood and metabolized into M1 and M3 by glucuronic acid enzyme. M1 and M3 are then further metabolized into M2 by glucuronic acid enzyme (figure 8).

3. Experimental

3.1 Instrumentation

HPLC analysis was carried out with a Waters 2690 pump and 996 PDA detector using a 5 μm Phenomenex RP-18 column (4.6 × 250 mm) maintained at 25°C. Detection was carried out at 320 nm with peak scanning from 200 to 400 nm (in 2 nm steps). The flow rate of the mobile phase was 0.8 ml min^{-1}. HPLC-MS was performed with an Agilent 1100 HPLC and PE SCIEX QSTAR MASS using a 5 μm Phenomenex RP-18 column (4.6 × 250 mm) maintained at 25°C, and the detection was set at 320 nm. Mass spectra were recorded using ESI in the positive mode with the ion spray voltage at 3800 VDS, source temperature 400°C, gas spray 1 at 0.38 MPa, gas spray 2 at 0.19 MPa, current gas at 40 psi (0.30 MPa), desolvent voltage 1 at 40 VDS, desolvent voltage 2 at 1.5 VDS, focus voltage at 215 VDS and scanning from mass 200 to 800. Preparative HPLC was performed on a Xinda pump made in China and a Shimadzu SPD-2A detector using a 5 μm Rainbow Kovasil RP-18 semi-preparative column (10 × 250 mm). An LD5-2A centrifuge (Beijing Medical Centrifuge Factory) and a Heidolph Diax 900 homogenizer were used.

3.2 Chemicals

Acetonitrile was of HPLC grade (Fisher, USA). Methanol was of GR grade and acetic acid and EtOAc were of analytical grade (Peking Chemical Factory). Physiological saline was purchased from Shandong Lingzi Pharmaceutical Company, and Tween-80 from Munchen, Germany. β-glucuronidase was purchased from Sigma.

Ferulic acid was provided by Professor Chen Ruoyun (>95% purity). It was dissolved in physiological saline at 50 mg ml^{-1} and the pH was adjusted to 6.5 by 1% NaOH solution; a small amount of Tween-80 helped dissolution.

3.3 Collection of animal and biological samples

Sixteen male Wistar rats (200 ± 20 g body weight) were provided by the Animal Center of Institute of Materia Medica. Before oral administration of ferulic acid, ten rats were fasted in

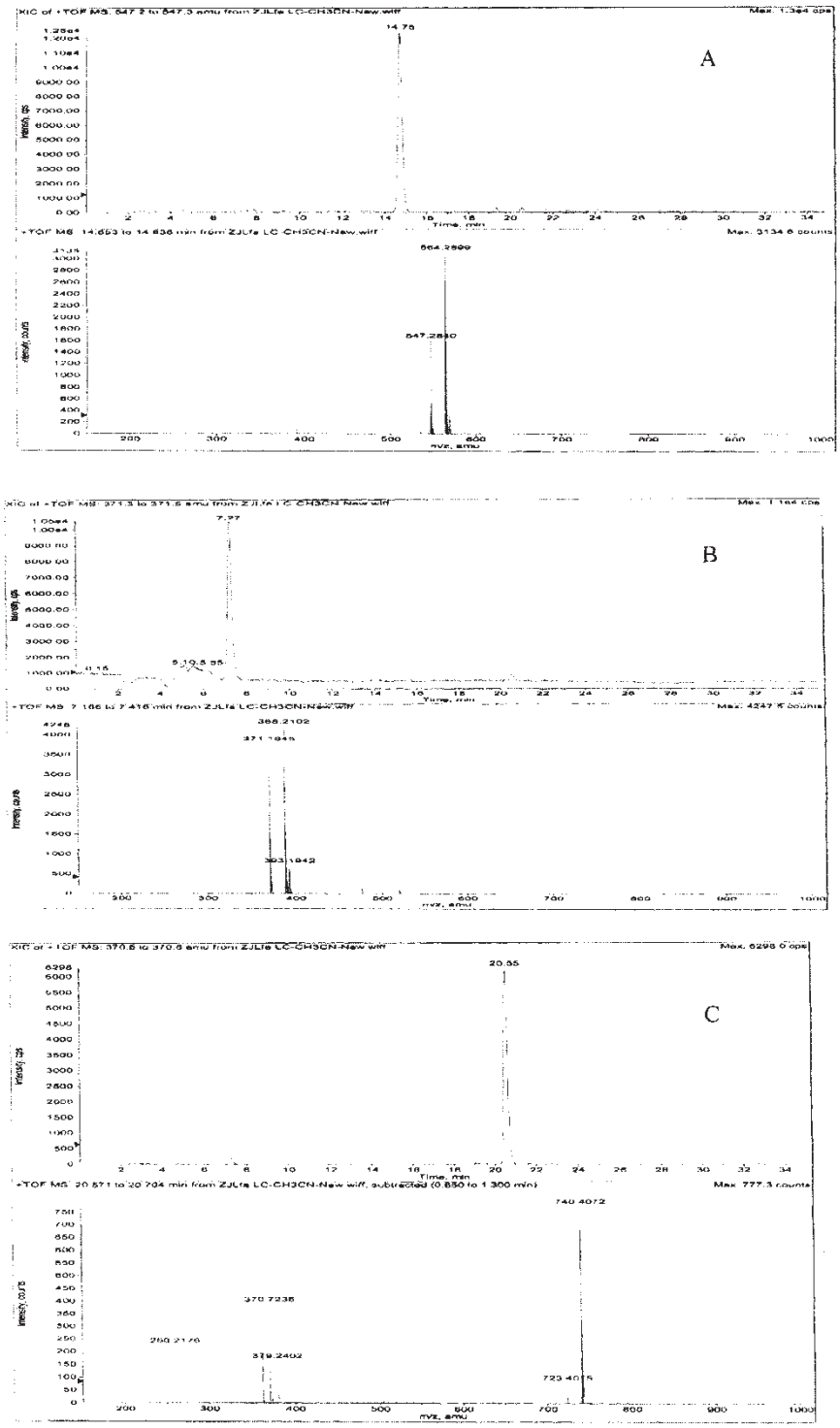

Figure 4. SIR and MS spectrum of the three metabolites. (A): M1, (B): M2 and (C): M3.

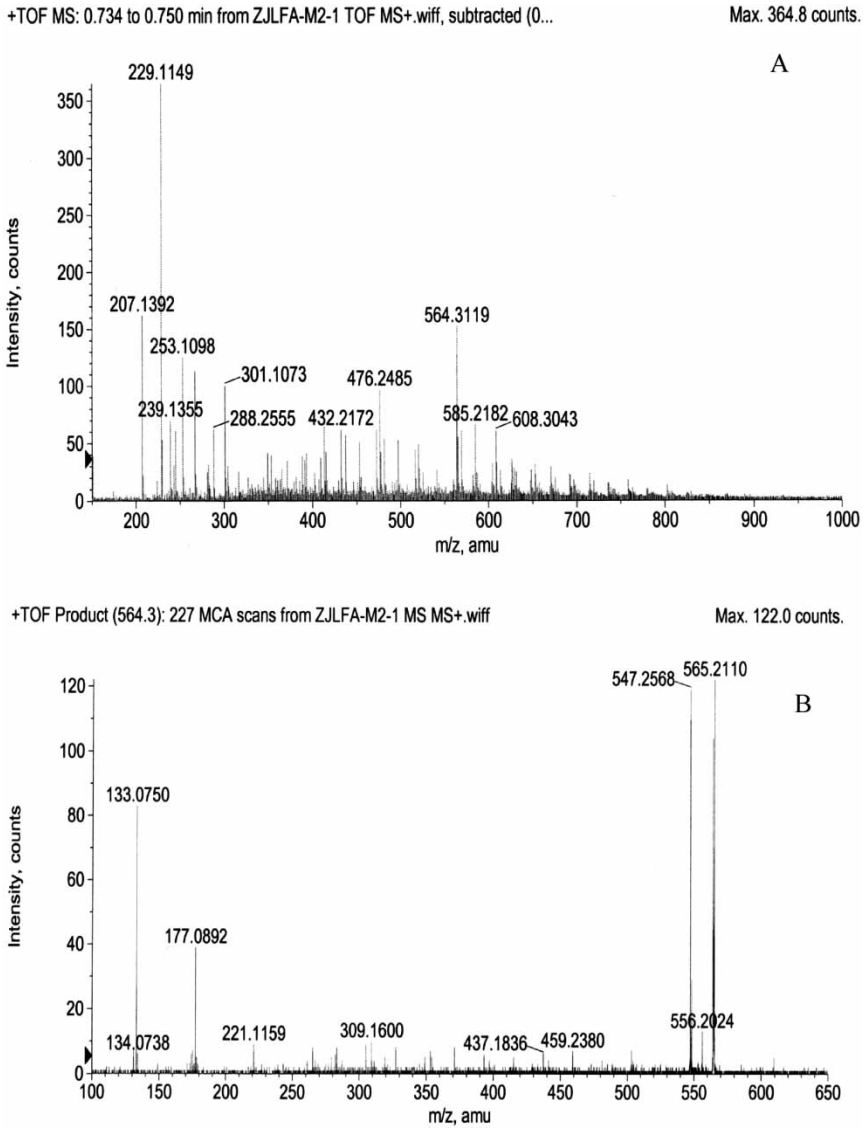

Figure 5. ESI$^+$-MS (A) and ESI$^+$-MS/MS (B) spectrum of M2.

a metabolic cage for 12 h, maintaining with physiological saline. Meanwhile blank urine and feces were collected. Five rats were orally administered with 200 mg (kg bodyweight)$^{-1}$ ferulic acid, and urine and feces were collected over 0–12 h and 12–24 h periods. Five rats were anaesthetized using ester and a polyethylene tube was inserted into their bile duct. After blank bile was collected for 4 h, the rats were orally administered with 200 mg (kg bodyweight)$^{-1}$ ferulic acid, and bile was collected over 0–12 h and 12–24 h periods.

Six rats were fasted for 12 h, maintaining with physiological saline. Three of them were administered with ferulic acid solution and the other three rats were used as the control. The rats' viscera (liver, spleen, kidney, stomach, heart and lung) were excised 2 h after oral administration of ferulic acid.

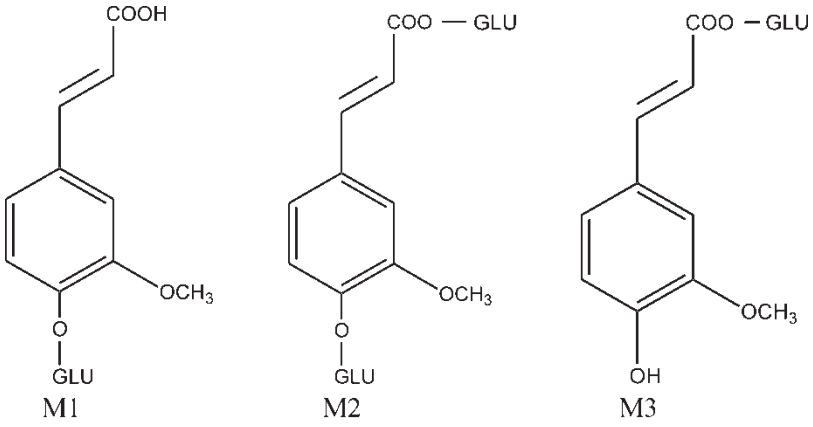

Figure 6. Structure of *in vivo* metabolites of ferulic acid.

3.4 Preparation of biological samples

The rats' urine or bile adjusted to pH 3.5 was subjected to ODS extraction (50 mg ODS per ml urine or bile) with H_2O adjusted to pH 3.5; H_2O and MeOH of equal volume were then used as eluents. MeOH fractions containing metabolites were evaporated to dryness under reduced pressure at 40°C and stored frozen until analyzed by HPLC as described below. Feces were ground completely in 10 × water and the mixture was then centrifuged. The resultant upper clear solution was subjected to ODS extraction as described above.

Rats' urine collected during 0–12 h was directly subjected to ODS extraction without acidification (50 mg ODS per ml urine or bile), with H_2O and MeOH of equal volume as subsequent eluents. MeOH fractions containing metabolites were evaporated to dryness under reduced pressure at 40°C and stored frozen until analyzed by HPLC-MS.

Rats' viscera (liver, spleen, kidney, stomach, heart and lung) were separately homogenized. The mixtures were centrifuged and the pH of the upper clear solution was adjusted to 3.5. They were then extracted by EtOAc of equal volume (3×). The EtOAc fractions were collected and evaporated to dryness under reduced pressure at 40°C and stored frozen until analyzed by HPLC.

3.5 Enzymatic hydrolysis

Urine was incubated in 0.1 M NaAc buffer (pH 5.1) with β-glucuronidase (2000 IU per ml urine) for 12 h at 37°C. The reaction was terminated by removing the reaction mixture from the incubator, and was subjected to ODS as described in the previous section.

3.6 Analysis of metabolites by HPLC-DAD and HPLC-MS

Each sample was dissolved in MeOH and then centrifuged at 3500 rpm. The resultant upper clear solution was then analyzed by HPLC-DAD and HPLC-MS with a 40-min gradient. Solvent A was 0.1% aqueous acetic acid solution and solvent B was acetonitrile. The gradient system was A–B (v/v) = 84/16 (0–5 min) A–B (v/v) = 76/24 (20 min) → A–B (v/v) = 60/40 (30 min) → A–B (v/v) = 84/16 (40 min).

3.7 Preparation of M2

Urine, after oral administration, over 0–24 h was treated by the method described in the preparation of biological samples. A sample dissolved in MeOH was subjected to HPLC

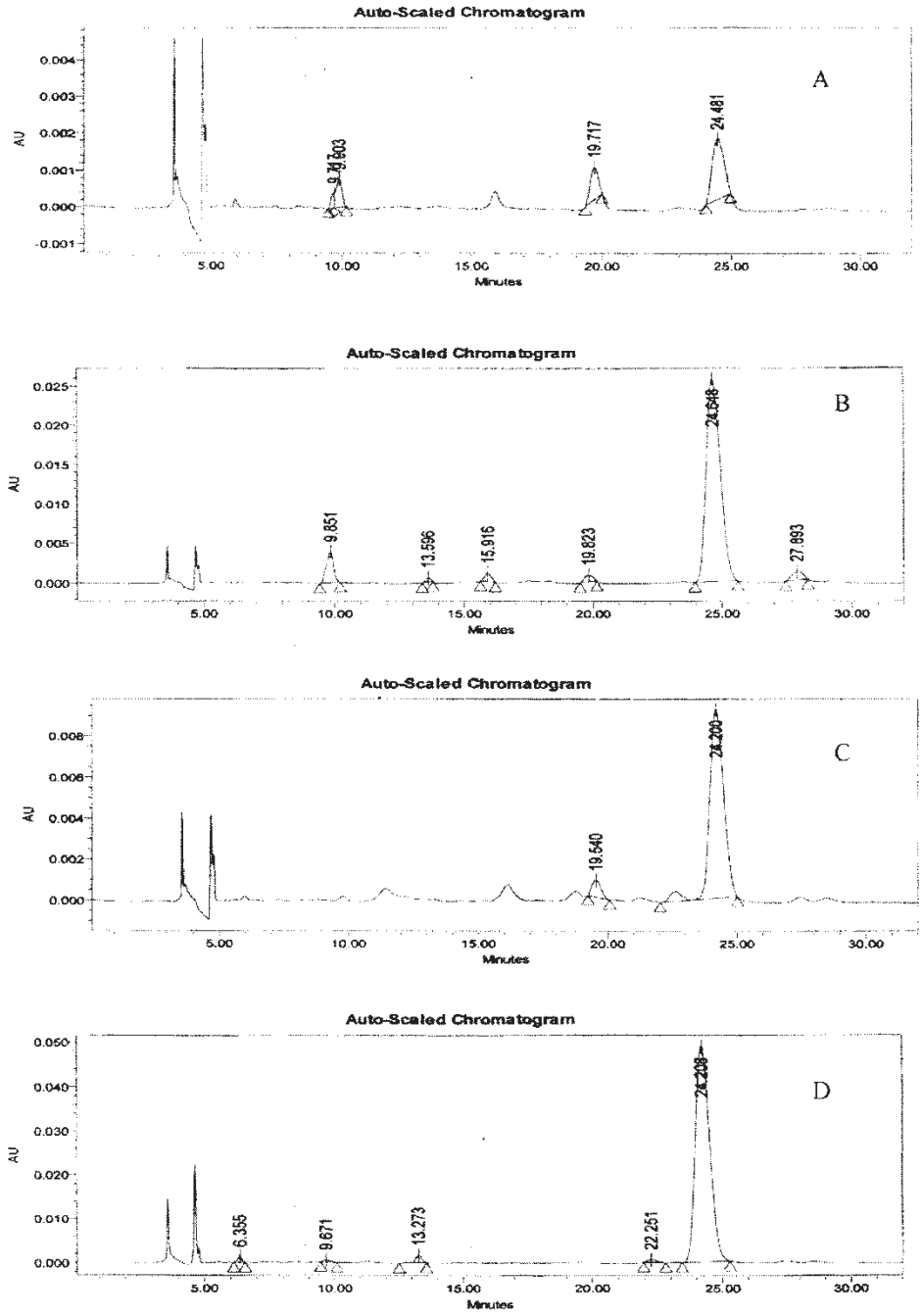

Figure 7. HPLC chromatogram of different visceras of rats after 2-h oral administration of ferulic acid. (A) Lung, (B) kidney, (C) spleen and (D) liver.

preparative column with isocratic elution. The mobile phase consisted of acetonitrile and 0.05% aqueous acetic acid (12/82) at a flow rate of 4.0 ml min^{-1}. The eluate fraction with a retention time of 15 min was collected and dried under reduced pressure at 40°C. The residue was then dissolved in MeOH and subjected to MS/MS.

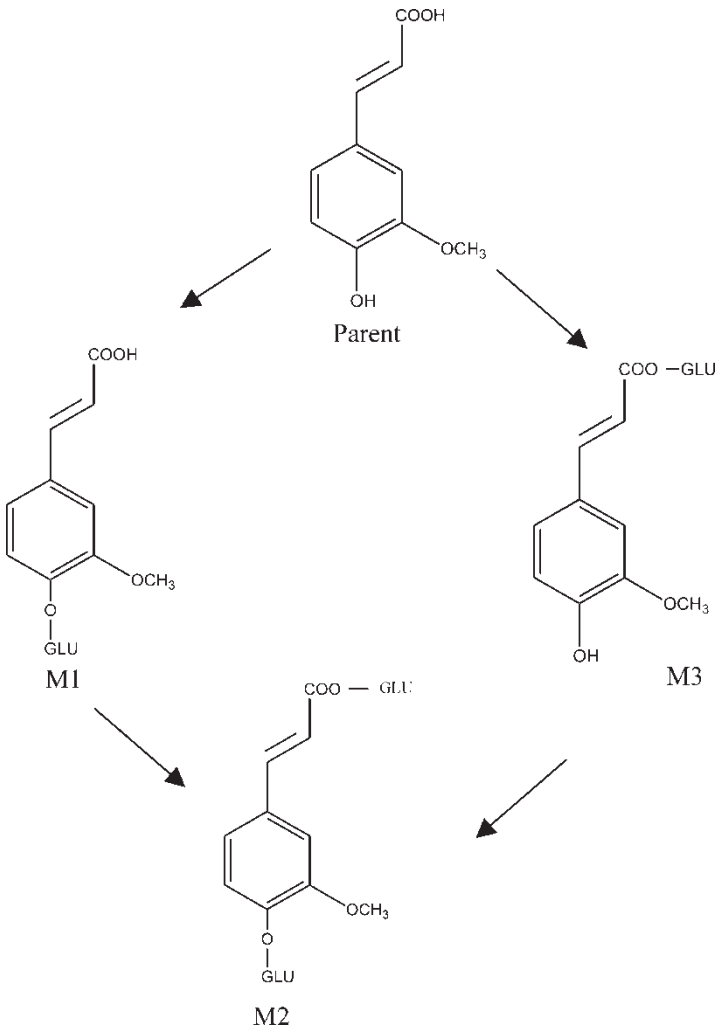

Figure 8. Metabolic pathway of ferulic acid in rats.

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