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Identification of metabolites of tectoridin in-vivo and in-vitro by liquid chromatography–tandem mass spectrometry

Yong Chen, Wei Song, Zhi H. Peng, Bao Y. Ge and Feng M. Han

Abstract

In this work, liquid chromatography–electrospray ionization tandem ion-trap mass spectrometry (LC–MS^n) was used to investigate the in-vivo and in-vitro metabolism of tectoridin. After oral administration of a single dose (100 mg kg^{-1}) of tectoridin to healthy rats, faeces and urine samples were collected for 0–48 h and 0–24 h, respectively. Tectoridin was also incubated with rat intestinal flora and rat liver microsomes. Samples from in-vivo and in-vitro metabolism studies were purified using a C_{18} solid-phase extraction cartridge, then separated using a reverse-phase C_{18} column with methanol/ water (30:70, v/v, adjusted to pH 10.0 with ammonia water) as mobile phase and detected by an on-line MS^n system. The structure of the metabolites was elucidated by comparing their molecular weights, retention times and full-scan MS^n spectra with those of the parent drug. The results revealed six metabolites of tectoridin in urine (tectorigenin, hydrogenated tectorigenin, mono-hydroxylated tectorigenin, di-hydroxylated tectorigenin, glucuronide-conjugated tectorigenin and sulfate-conjugated tectorigenin); three metabolites in faeces (tectorigenin, di-hydroxylated tectorigenin and sulfate-conjugated tectorigenin); one metabolite in the intestinal flora incubation mixture (tectorigenin), and four in the liver microsomal incubation mixture (tectorigenin, hydrogenated tectorigenin, mono-hydroxylated tectorigenin and di-hydroxylated tectorigenin). Except for tectorigenin, all other metabolites of tectoridin are reported for the first time.

Introduction

Tectoridin is the main active component of *Belamcanda chinensis*, which is widely used in Chinese traditional medicine. Tectoridin possesses a number of physiological activities, including anti-infective, antitussive, expectorant, antibacterial, antiviral and analgesic effects (Yoshiyasu et al 1993; Kim et al 1999; Qin et al 2003; Qin et al 2004; Kang et al 2005). By contrast with the comprehensive investigations for therapeutic effects, few data are available on the metabolites of tectoridin. To our knowledge, only two papers have described the biotransformation of tectoridin in intestinal bacteria, in which tectoridin was transformed into tectorigenin by human intestinal bacteria (Bae et al 1999; Shin et al 2006). No other metabolites of tectoridin have been reported as yet. Liquid chromatography–electrospray ionization (ESI) tandem ion-trap mass spectrometry (LC–MS^2) has high sensitivity and selectivity, and has become a powerful tool for the identification of drug metabolites in biological materials (Rudewicz et al 1986; Yu et al 1999; Lam & Ramanathan 2002; Chan et al 2003). Because metabolites can retain the basic structural features of the parent drug after biotransformation, multistage MS of each metabolite can provide abundant structural information, and the structure of metabolites may be characterized rapidly using the multistage MS fragmentation pathway of the parent drug as a structural template, even when no metabolite standards are available (Kerns et al 1997; Dong et al 2002; Gangl et al 2002; Chan et al 2003; Appolonova et al 2004; Chung et al 2004).

In this work, a sensitive and specific LC–MS^n method was developed to identify the metabolites of tectoridin in rat urine and faeces after oral administration of a single dose (100 mg kg^{-1}) of tectoridin to healthy rats, along with the metabolites of tectoridin after incubation with rat intestinal flora and liver microsomes in-vitro. The aim of the work was to clarify the in-vivo and in-vitro metabolic pathways of tectoridin in the rat.

Hubei Province Key Laboratory of Biotechnology of Chinese Traditional Medicine, Hubei University, 430062, P. R. China

Yong Chen, Wei Song, Zhi H. Peng, Bao Y. Ge, Feng M. Han

Correspondence: Y. Chen, College of Life Science, Hubei University, Wuhan, 430062, P. R. China. E-mail: cy101610@npc.gov.cn

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Materials and Methods

Reagents and chemicals

Tectoridin was extracted and purified from rhizomes of *Iris tectorum* by HPLC; the purity of tectroidin was >98% (Liu et al 2006). Methanol was LC grade (Fisher Chemical Co., Inc, CA, USA). Water was deionized and double distilled. Other reagents were analytical grade.

Intestinal incubations

Anaerobic culture solutions were prepared as follows (Hattori et al 1985): 37.5 mL solution A (0.78% K_2HPO_4), 37.5 mL solution B (0.47% KH_2PO_4, 1.18% NaCl, 1.2% $(NH_4)_2SO_4$, 0.12% $CaCl_2$ and 0.25% $MgSO_4 \cdot H_2O$) and 50 mL solution C (8% Na_2CO_3) were mixed with 0.5 g L-cysteine, 2 mL 25% L-ascorbic acid, 1 g beef extract, 1 g tryptone and 1 g nutrient agar and dissolved in 1 L distilled water; the pH was adjusted to 7.4 with 2 M HCl.

Intestinal incubation experiments were performed in anaerobic incubation bags (AnaeroPouch Anaero 08G05A-23, Mitsubishi Gas Chemical Company Inc., Tokyo, Japan) using anaerobic-generating bags (Mitsubishi Gas Chemical Company Inc.).

Analysis

$LC–MS^n$ experiments were performed on an LCQ Duo quadrupole ion-trap mass spectrometer (Thermo-Finnigan Corp., San Jose, CA, USA) with an Agilent 1100 Series G1311A Quat pump and a G1313A autosampler, using negative electrospray as the ionization process. Commercial software (Xcalibur version 1.2, Thermo-Finnigan) was used for system operation and data collection.

The mobile phase consisted of methanol and water (30:70 v/v, adjusted to pH 10.0 with ammonia water) and delivered at a flow rate of 0.2 mL min^{-1}. Before use, the mobile phase was filtered through a 0.45 μm filter. The injection volume was 20 μL.

The operating conditions for the mass measurements were optimized by direct infusion into the MS of 50 μL 10 μg mL^{-1} tectoridin solution (in mobile phase) and chosen as follows: ion-spray voltage −60 kV; capillary voltage −37 V; heated capillary temperature 200°C; sheath gas flow rate 80 arbitrary units; auxiliary gas flow rate 20 arbitrary units. The other parameters, including the voltages of the octapole offset and tube lens offset, were also optimized to achieve the maximum abundance of the ions of interest by the automatic tuning procedure. By isolating the molecular ions of metabolites in the ion trap, the product ion spectra were obtained by collision-induced dissociation (CID). The energy of CID was 38%. We used the selected reaction monitoring mode to determine the retention times of the parent drug and its metabolites. Data acquisition was performed in full scan LC–MS and tandem MS^n modes.

In-vitro study

Animals were managed according to the rules and regulations of the Institutional Animal Care and Use Committee at the Hubei University, which also approved the experimental protocol.

Six Wistar rats (male and female, 180 ± 5 g, Hubei Experimental Animal Research Center, China) were housed in metabolic cages for the collection of urine and faeces. The rats were fasted for 24 h but with access to water, and then given a single dose of tectoridin (100 mg kg^{-1}) by gavage. Faeces from individual rats were collected for 0–48 h, and urine for 0–24 h. The samples were stored at −20°C until analysis.

The 48 h faeces sample was homogenized with water and then centrifuged at 5000 *g* for 10 min. A 1 mL aliquot of the supernatant sample was loaded onto a C_{18} solid-phase extraction (SPE) cartridge (3 mL/200 mg (v/w); AccuBond, Agilent) preconditioned with 2 mL methanol and 1 mL water. The SPE cartridge was then washed with 2 mL water to elute endogenous impurities, followed by 1 mL methanol to elute the analytes. The methanol eluent was centrifuged at 10 000 *g* for 10 min and a 20 μL aliquot used for $LC–MS^n$ analyses.

The 24 h urine sample was also centrifuged at 5000 *g* for 10 min. A 1 mL aliquot of the supernatant was loaded onto a C_{18} SPE, preconditioned with 2 mL methanol and 1 mL water. The SPE cartridge was then washed with 2 mL water and the analytes eluted with 1 mL methanol. The elution solutions were centrifuged at 10 000 *g* for 10 min and a 20 μL aliquot used for $LC–MS^n$ analyses.

In-vitro study

Metabolism by rat intestinal bacteria

Fresh faeces obtained from Wistar rats were immediately homogenized with a glass rod in anaerobic culture solution in the ratio 1:4 (w/v). Then, 1 mL of the homogenized suspension was mixed with 4.0 mL anaerobic culture solution. Finally, 1 mL tectoridin (1 mg mL^{-1}) was added to 5.0 mL of the above intestinal flora culture solution and cultured in anaerobic conditions in a shaking water bath at 37°C for 24 h. After the incubation, 2 mL methanol was added to the incubation solution, which was then centrifuged at 5000 *g* for 10 min. The supernatant was decanted and evaporated at 37°C under a nitrogen stream. The residue was resuspended in 1 mL mobile phase and centrifuged at 10 000 *g* for 10 min. A 20 μL aliquot of the supernatant was used for $LC–MS^n$ analysis.

Metabolism by rat liver microsomes

Wistar rats were fasted overnight before being killed. Minced liver tissue was homogenized in 0.1 mol L^{-1} phosphate-buffered saline (PBS) (pH 7.4) and centrifuged at 10 000 *g* for 10 min. The supernatant was decanted and centrifuged at 100 000 *g* for 60 min to give the microsome fraction. The liver microsomes were resuspended in PBS and immediately stored at −80°C. Tectoridin and the liver microsome suspensions were added to PBS at concentrations of 200 μg mL^{-1} tectoridin and 1.0 mg mL^{-1} total protein. The incubation was started by the addition of NADPH (final concentration 1.0 mmol L^{-1}) at 37°C under agitation, and terminated after 1 h by adding twice the volume of ice-cold methanol. Precipitated protein was removed by centrifugation at 10 000 *g* for 10 min

at 4°C. The supernatant was evaporated at 37°C under a nitrogen stream. The residue was resuspended in 1 mL mobile phase and then centrifuged at 10 000 *g* for 10 min. A 20 μL aliquot of the supernatant was used for LC–MS^n analysis.

Results and Discussion

LC–MS^n analysis of tectoridin

Under the LC–MS^2 conditions, tectoridin eluted at 2.34 min (Figure 1A). The full-scan mass spectrum of tectoridin indicated its deprotonated molecule $[M\text{-}H]^-$ at m/z 461 (Figure 1B). The MS^2 of the molecular ion at m/z 461 led to six main product ions, at m/z 446, 299, 284, 283, 256 and 240 (Figure 1C). The most abundant fragment ion of m/z 299, formed by the loss of Glu (162 Da) from the molecular ion, led to four main MS^3 fragment ions, at m/z 284, 283, 256 and 240 (Figure 1D). The MS^n fragmentation pathway of tectoridin is shown in Figure 2. The productions and the corresponding neutral fragment loss of the parent drug provided a sound basis for structural elucidation of the metabolites.

In-vivo metabolism study

Identification of metabolites in rat urine

Since the fragment information of tectoridin and its metabolites were more and higher in negative-ion mode than those in positive-ion mode, MS experiments were performed in negative-ion mode. After purification of the in-vivo and in-vitro samples, no endogenous compounds were found to interfere with the MS^n spectra analysis of the metabolites.

To identify the metabolites, the possible structures were speculated from the structure of tectoridin. By comparing the full-scan mass spectrum of the rat urine sample after administration of 100 mg kg^{-1} tectoridin with that of control blank rat urine, parent drug and its six possible metabolites were found in the purified urine sample. Their molecular ions $[M\text{-}H]^-$ were at m/z 299, 301, 315, 331, 379 and 475, and the MS^n data of them were obtained by CID of their molecular ions $[M\text{-}H]^-$ (Figure 3). The MS^2 and MS^3 spectra of the molecular ion at m/z 461 (M0) were the same as those of tectoridin. Therefore, M0 was the unchanged parent drug.

The molecular ion of M1 (m/z 299, T = 2.48 min) was 162 Da less than that of M0, and led to four main characteristic fragment ions at m/z 284, 283, 256 and 240 (Figure 3A), which was the same as the MS^3 spectrum of M0 at m/z 461→299. Furthermore, the MS^3 of M1 (m/z 299→284) produced the fragment ion at m/z 266, 256 and 240 (Figure 3B). The fragment ion at m/z 266 was formed by the loss of H_2O (18 Da) from the ion at m/z 284. So M1 can be confirmed as the de-Glu group product of tectoridin, and it was named as tectorigenin.

The molecular ion of M2 (m/z 301, T = 2.35 min) and its MS^2 ions at m/z 286, 285, 258 and 242 (Figure 3C) were all 2 Da more than the molecular ion of M1 and its main product ions at m/z 284, 283, 256 and 240, respectively. The MS^3 of M2 (m/z 301→286) led to three main

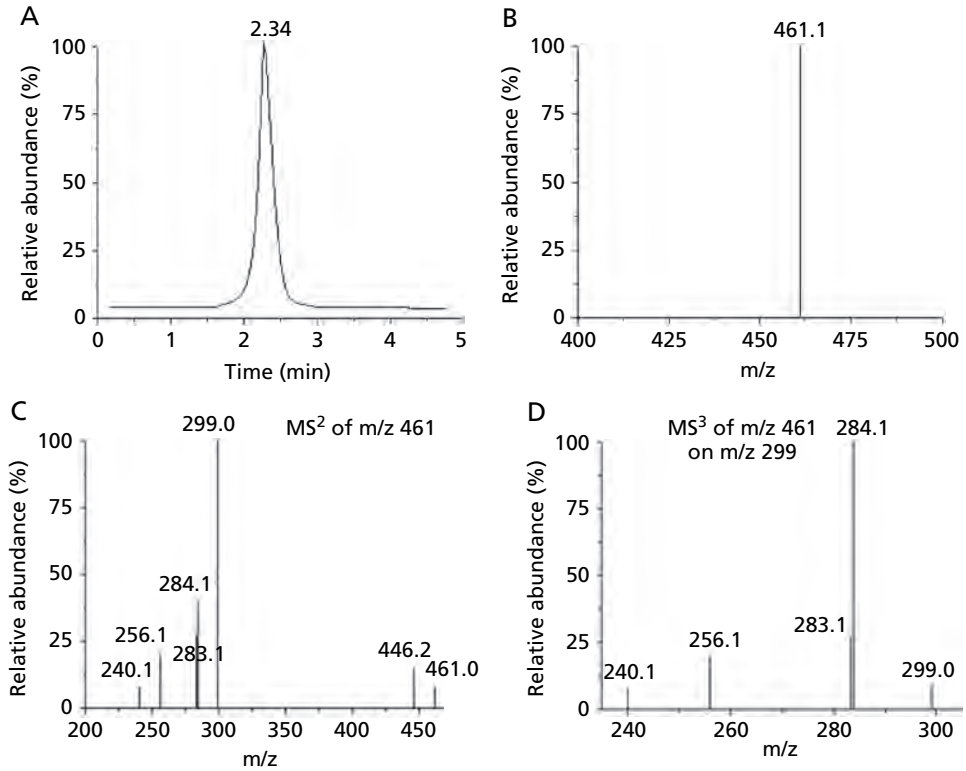

Figure 1 The Chromatogram (A) and MS (B), MS^2 (C) and MS^3 (D) spectra of tectoridin.

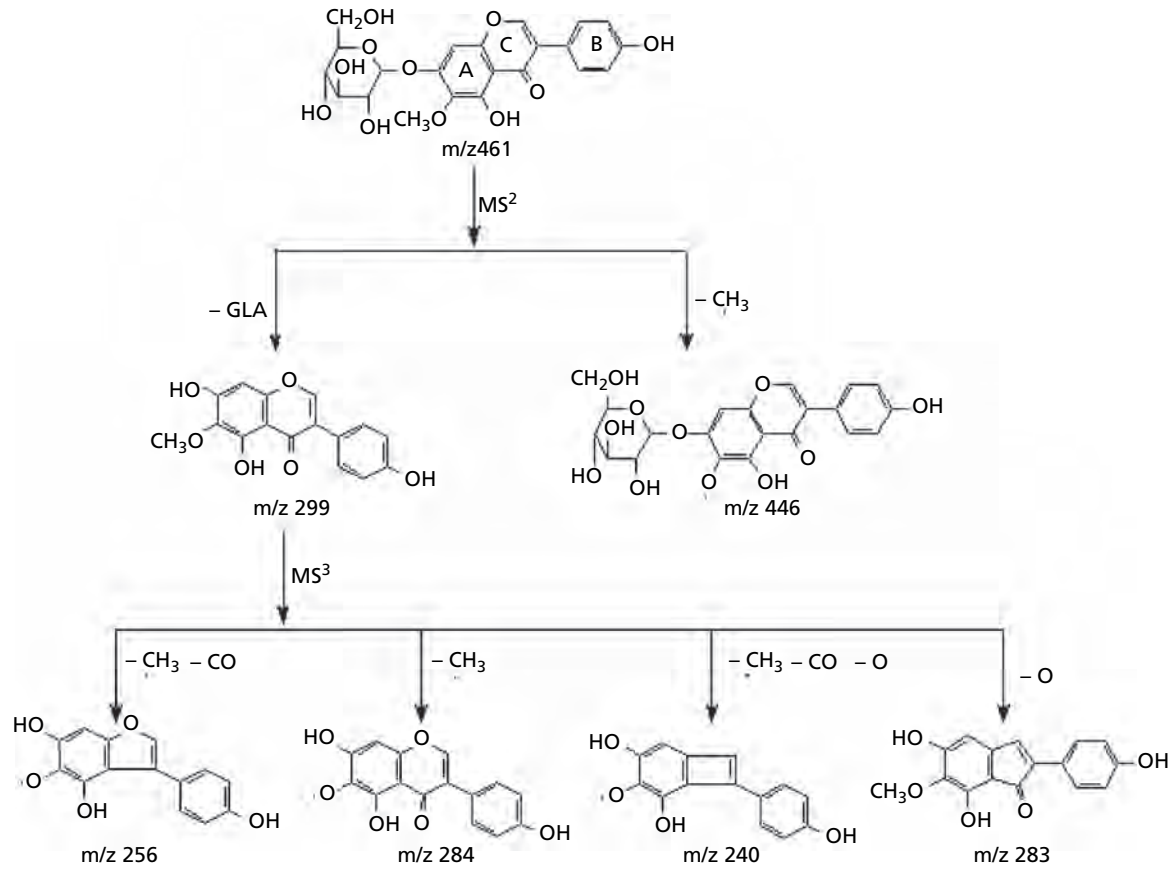

Figure 2 The proposed fragmentation pathway of tectoridin.

fragment ions at m/z 268, 258 and 242 (Figure 3D), which were also 2 Da more than those of MS^3 fragment ions of M1 (m/z 299→284), respectively. These results indicate that M2 was the hydrogenated product of tectorigenin, and the reduction position was located at the C-ring of tectorigenin.

The molecular ion of M3 (m/z 315, T = 2.24 min) and its MS^2 ions at m/z 300, 299, 272 and 256 (Figure 3E) were all 16 Da more than the molecular ion of tectorigenin and its daughter ions at m/z 284, 283, 256 and 240, respectively. The MS^3 of M3 (m/z 315→300) yields three main fragment ions, at m/z 282, 272 and 256 (Figure 3F), which were also 16 Da more than the MS^3 fragment ions of M1 (m/z 299→284), respectively. Thus, M3 may be the monohydroxylated product of M1, at the B-ring of tectorigenin.

The molecular ion of M4 (m/z 331, T = 2.43 min) and its MS^2 fragment ions at m/z 316, 315, 288 and 272 (Figure 3G) were all 32 Da more than the molecular ion of M1 and its MS^2 fragment ions at m/z 284, 283, 256 and 240, respectively. The MS^3 of M4 (m/z 331→316) produced three main fragment ions, at m/z 298, 288 and 272 (Figure 3H), which were also all 32 Da more than the MS^3 fragment ions of M1 (m/z 299→284), respectively. These results indicate that M4 may be the di-hydroxylated product of M1, the hydroxylated positions being on the B-ring of tectorigenin.

The molecular ion of M5 (m/z 379, T = 2.31 min) led to three main product ions at m/z 364, 299 and 79 (Figure 3I). The most abundant product ion at m/z 299 was formed by loss of the neutral fragment SO_3 (80 Da). The MS^3 spectrum of M5 (m/z 379→299) (Figure 3J) was the same as the MS^2 spectrum of the molecular ion of M1. In addition, the daughter ion at m/z 79 was present in the MS^2 spectrum of M5. These results indicate that M5 was the sulfate-conjugated product of M1. The sulfate was conjugated on the B-ring of tectorigenin.

The molecular ion of M6 (m/z 475, T = 2.20 min) produced three main product ions, at m/z 460, 299 and 175 (Figure 3K). The abundant daughter ion at m/z 299 was produced by loss of the neutral fragment of 176 Da, and the MS^3 spectrum of M6 (m/z 475→299) (Figure 3L) was the same as the MS^2 spectrum of M1. Furthermore, the daughter ion at m/z 175 was present in the MS^2 spectrum of M6, along with the appearance of the fragment ion at m/z 113 in the MS^3 spectrum of M6 (m/z 475→175) (Figure 3M). This fragmentation behaviour (m/z 475→175→113) is the cleavage feature of glucuronide conjugates (Chen et al 1998; Gu et al 1993). All the results indicate that M6 was the glucuronide-conjugated product of M1, conjugated at the B-ring of tectorigenin.

Identification of metabolites in rat faeces

The full-scan mass spectrum of rat faeces after administration of tectoridin was compared with that of a sample

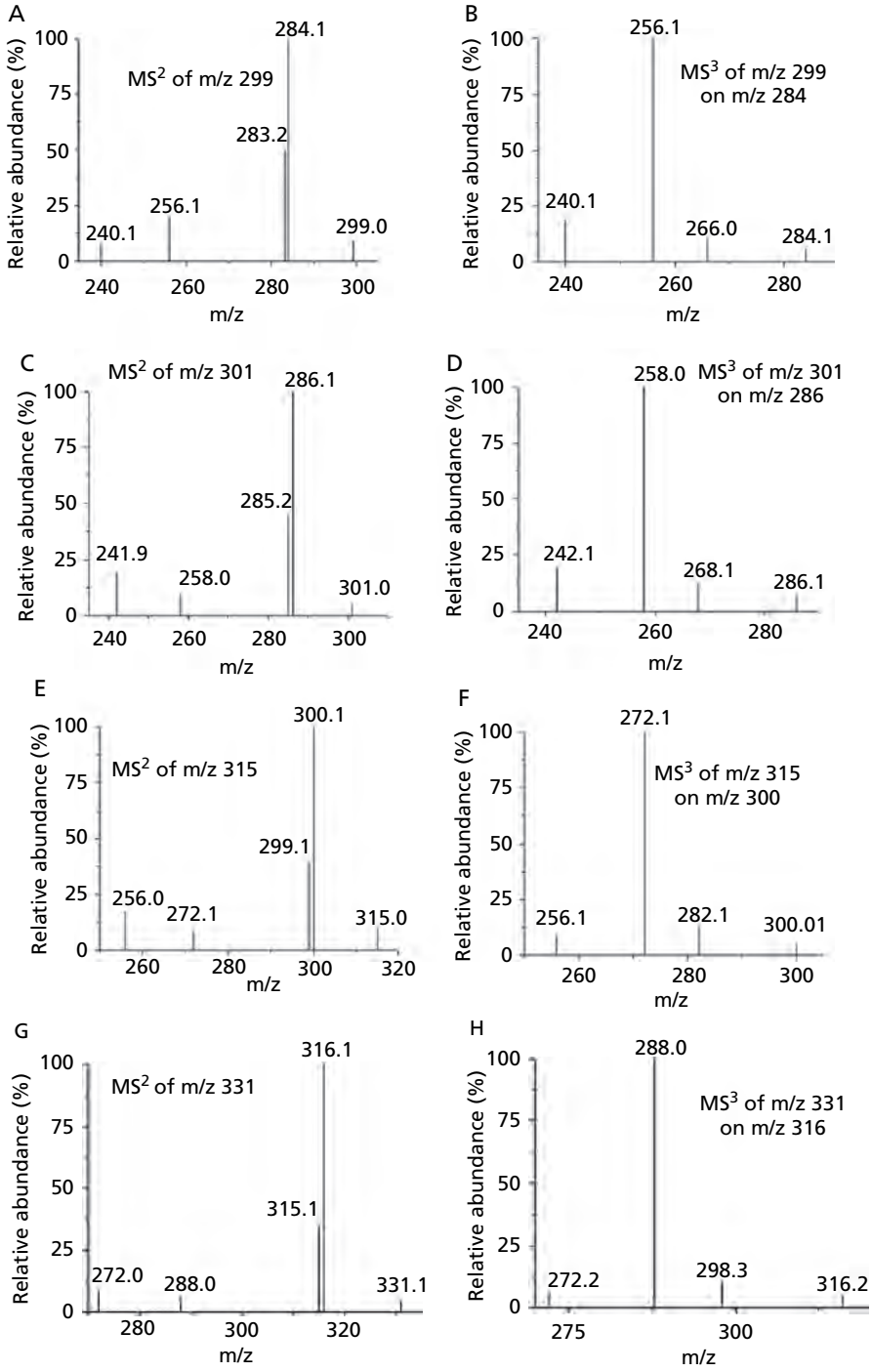

Figure 3 MS^2 and MS^3 spectra of the metabolites of tectoridin in-vivo and in-vitro. A, MS^2 of m/z 299; B, MS^3 of m/z 299 on m/z 284; C, MS^2 of m/z 301; D, MS^3 of m/z 301 on m/z 286; E, MS^2 of m/z 315; F, MS^3 of m/z 315 on m/z 300; G, MS^2 of m/z 331; H, MS^3 of m/z 331 on m/z 316; I, MS^2 of m/z 379; J, MS^3 of m/z 379 on m/z 299; K, MS^2 of m/z 475; L, MS^3 of m/z 475 on m/z 299; M, MS^3 of m/z 475 on m/z 175. *(Continued)*

of blank faeces to find the possible metabolites. These compounds were then analysed by LC–MS^n. The retention times, changes in observed mass and spectral patterns of product ions were compared with those of tectoridin to identify metabolites and elucidate their structures. Based on the method described above, the parent drug and its three metabolites M1 (m/z 299), M4 (m/z 331) and M5 (m/z 379) were found in rat faeces after administration of a single dose of tectoridin (100 mg kg^{-1}) to healthy rats.

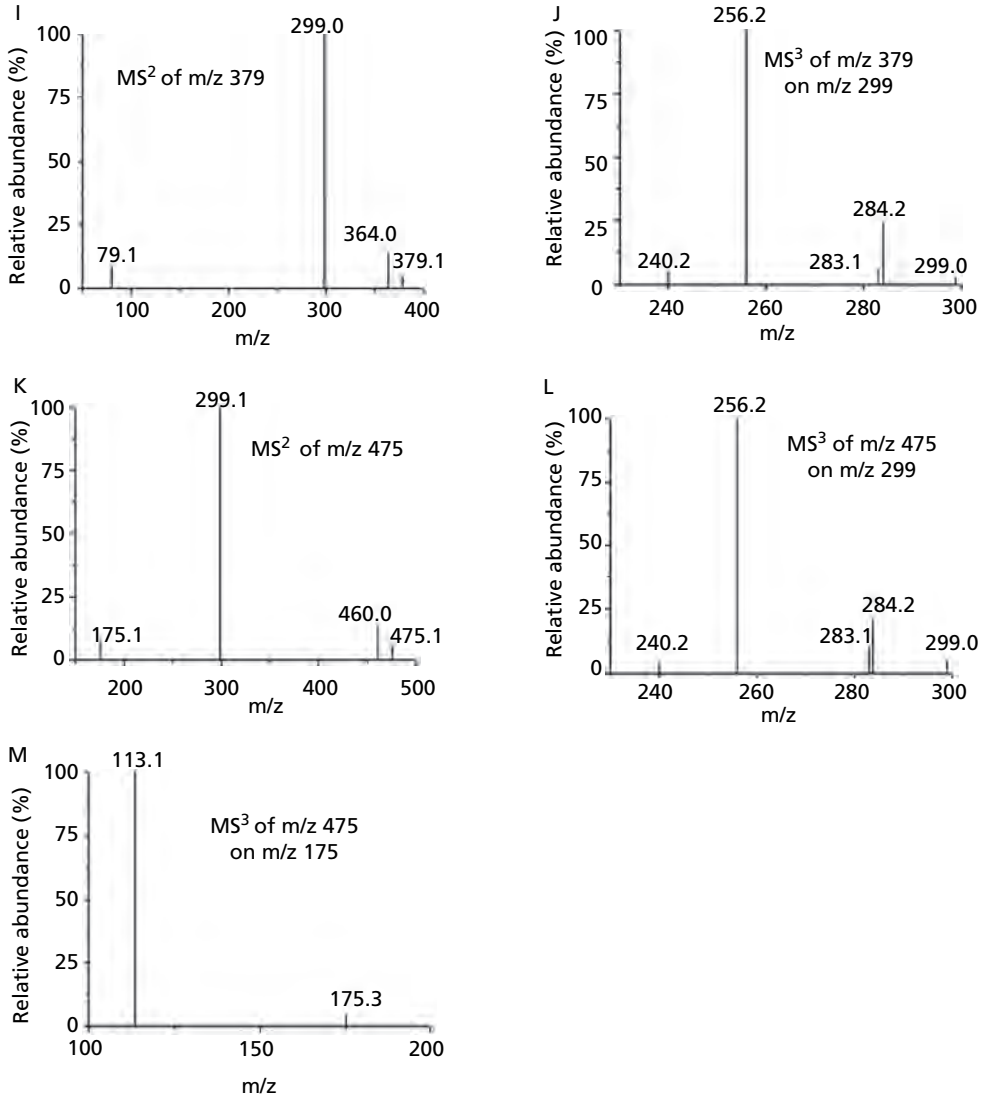

Figure 3 *(Continued).*

In-vitro metabolism study

When tectoridin was incubated with intestinal flora (from fresh rat faeces), only one metabolite, named as tectorigenin (M1), was detected in addition to the parent drug.

Four metabolites were detected in liver microsomal incubation solutions: tectorigenin, hydrogenated tectorigenin, mono-hydroxylated tectorigenin and di-hydroxylated tectorigenin (M1, M2, M3 and M4).

Conclusions

Tandem MS provides much greater specificity than single-stage MS, allowing truly selective detection. Such specific detection provides greater confidence in assays and reduces the requirement for efficient separations. Although ESI techniques that are typically employed in LC–MS^n provide less structural information than is available from electron ionization gas chromatography/MS, ESI ion-trap multistage MS can also provide abundant structural information about the analyte. In this work, a sensitive and specific LC-coupled ESI ion-trap multistage MS method was developed for identification of tectoridin and its metabolites in-vivo and in-vitro. The results revealed that six metabolites of tectoridin (tectorigenin, hydrogenated tectorigenin, mono-hydroxylated tectorigenin, di-hydroxylated tectorigenin, glucuronide-conjugated tectorigenin and sulfate-conjugated tectorigenin) existed in rat urine, and three metabolites (tectorigenin, di-hydroxylated tectorigenin and sulfate-conjugated tectorigenin) in rat faeces. The proposed major metabolic pathways of tectoridin in-vivo are shown in Figure 4. Four metabolites were found in liver microsomal incubations and only one metabolite in intestinal flora incubations. Except for tectorigenin, all other metabolites of tectoridin are reported for the first time. Although the hydroxylated and conjugated position of the parent drug cannot been confirmed using this method alone, this work is valuable for understanding the metabolism of tectoridin in-vivo and in-vitro.

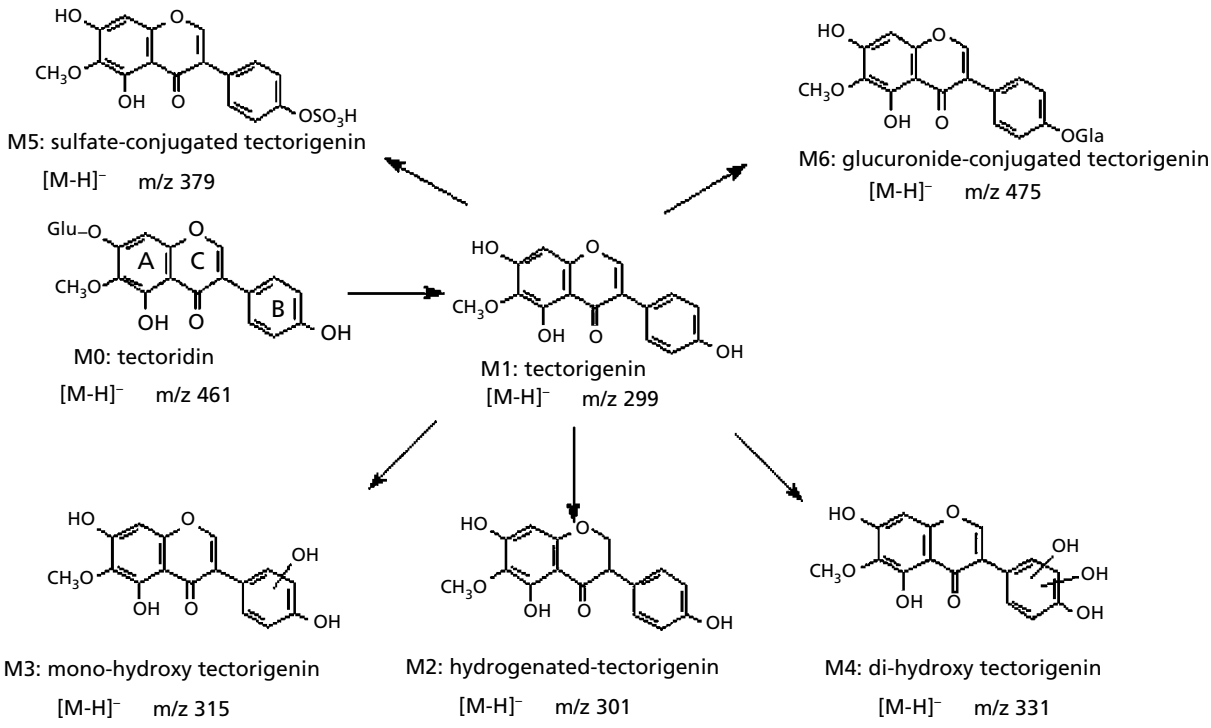

Figure 4 Proposed metabolic pathway of tectoridin in the rat in-vivo. Gla = glucuronic acid.

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