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An approach to identifying sequential metabolites of a typical phenylethanoid glycoside, echinacoside, based on liquid chromatography–ion trap-time of flight mass spectrometry analysis

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ABSTRACT

Metabolite identification for the compounds that undergo multiple and sequential metabolism is still a great challenge. Echinacoside (ECH), a typical phenylethanoid glycoside, contains multiple unstable chemical bonds and high reactive functional groups which are susceptible to multiple pathways of degradation and metabolism, leading great difficulties for its metabolite identification. This study proposed a novel approach for rapidly identifying the complicated and unpredictable metabolites of ECH, based on the powerful liquid chromatography hybrid ion trap and time of flight mass spectrometry (LC/MS-IT-TOF) analysis. Four degradation products were rapidly identified via the "fragmentation–degradation" comparisons. Five phase I and phase II metabolites of the degradation products were rapidly characterized via the crossover mass differences comparisons of their quasi-molecular ions with the potential precursors. Four direct phase I and phase II metabolites of the parent compound were identified by the mass differences analysis of the molecular ions between metabolites and the parent compound. Multiple stages of fragmentation patterns were used to confirm the metabolites characterizations. This study provides a novel approach to characterizing the complicated metabolites, and would be widely applicable for the metabolite identification of natural products.

1. Introduction

Phenylethanoid glycoside is a class of polyphenolic compound distributed in many plants. They are reported to posses cytostatic [1], cardioactive [2], hepatocyte protective [3], antibacterial, antioxidative [4], neuroprotective [5] effects and so on. In particular, echinacoside (ECH), a representative phenylethanoid glycoside contained in Herb Cistanchis [6], *Echinacea angustifolia* and *E. pallida* roots have been the best-selling herbal medicine in the United States and Europe as the immunostimulant [7]. It has been also observed to have various kinds of other pharmacological activities including antioxidant [8–10], neuroprotective [11] and anti-inflammatory [12].

In spite of the well defined pharmacological activities of phenylethanoid glycoside, very less is known about its pharmacokinetic and metabolic characteristics. Previous studies showed that no phenylethanoid glycoside was detected in human plasma after echinacea tablets ingestion [13] and the bioavailability of echinacoside in rat was only about 0.83% [14], suggesting poor membrane permeability and/or extensive presystemic metabolism.

The chemical structure of ECH is composed of three chemical moieties including cafffeic acid (CA), phenylethanoid aglycone (PhA, 3,4-dihydroxyphenethyl alcohol, hydroxytyrosol), and sugar. The chemical bonds linking these moieties and the multiple hydroxyl groups in ECH structure are susceptible to multiple and sequential routes of hydrolysis, phase I (oxidation and reduction) and/or phase II (glucuronic acid, sulfate, and methyl conjugation) metabolism. Thus, we hypothesized that ECH may be susceptible to extensive and multiple metabolism. In a recent study [15], eight phase II metabolites (methyl and glucuronic acid conjugations) of ECH were identified from the rat bile samples. Although this study resulted in an unambiguous identification of eight metabolites of ECH by using NMR, we hypothesized that there should have much other metabolites failed to be identified, in view of the characteristics of ECH chemical structure. The present study was thus aimed to characterize ECH metabolites from rat urine and feces as many as possible.

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Table 1
Accurate mass measurements of pseudo-molecular and fragment ions of echinacoside.

Pseudo-molecular ion (PI)			Fragment ion (MS^2)		
Measured m/z (% base peak, mDa[a], ppm[b])	Calculated m/z	Formula	Measured m/z (% base peak, mDa, ppm)	Calculated m/z	Fomula
392.1228[c] (100.00, 1.0, 2.55)	392.1218[c]	$C_{35}H_{46}O_{20}$	135.0458 (58.27, 0.6, 4.44)	135.0452	$C_8H_8O_2$
785.2481 (5.39, −2.9, 3.69)	785.2510	$C_{35}H_{46}O_{20}$	161.0266 (24.47, 2.2, 13.66)	161.0244	$C_9H_6O_3$
			179.0349 (100.00, −0.1, 0.56)	179.0350	$C_9H_8O_4$
			311.0931[c] (20.77, −2.3, 7.39)	311.0954[c]	$C_{29}H_{36}O_{15}$
			315.1062 (1.3, −2.3, 7.30)	315.1085	$C_{14}H_{20}O_8$
			461.1656 (1.41, −0.6, 1.30)	461.1662	$C_{20}H_{30}O_{12}$
			477.1583 (3.12, −3.1, 6.50)	477.1614	$C_{20}H_{30}O_{13}$
			623.2182 (25.88, −1.1, 1.77)	623.2193	$C_{26}H_{40}O_{17}$

[a] Milli-Dalton, differences between the measured and calculated values.
[b] Differences between the measured and calculated values.
[c] $[M-2H]^{2-}$ ions in negative scan mode, while others were $[M-H]^-$ ions.

Despite the recent advancement of various analytical tools, the metabolite identification for the compounds undergoing multiple and unpredictable metabolism from the biological matrix remains still a great challenge. In the past few years, mass spectrometry (MS) coupled with chromatographic separation has become a powerful and frequently used technique for metabolite identification. For their low sensitivity in full scan mode, LC/MS or LC/MS/MS are challenged for complex biological samples containing many compounds [16]. In contrast, LC–TOF/MS characterized with a wide mass range, sensitivity in full scan mode, the high resolving power, and accurate mass measurement, enables to the powerful identification of non-target compounds at trace level [17,18]. In addition, LC–IT/MS is also a useful tool for identifying unknown compounds because of its ability to gain abundant fragment ions [19]. The LC/MS-IT-TOF and LC–QTOF/MS, a combination of these two techniques can provide structural information from fragmentation as well as elemental compositions from accurate mass measurement, which represents a powerful tool to analyze unknown compounds of trace levels in complex matrices [20–27].

The present study was thus designed to detect and identify the metabolites of ECH in urine and feces based on the powerful LC/MS-IT-TOF analysis. A generally applicable approach to identifying the complicated metabolites including the degradation products, the phase I/phase II metabolites of the parent compound and its degradation products has been proposed in this study.

2. Experimental

2.1. Chemicals and reagents

Echinacoside (powder, purity 96.4%) was separated and purified from an ethanol extract of Cistanche tubulosa (Schenk) R. Wight [14]. Verbascoside (powder, purity 98.8%) was purchased from Chengdu Push Bio-Technology Co., Ltd. (Chengdu, Sichuan, China). 3,4-Dihydroxyphenethyl alcohol (oil, purity 98.5%) was from Chengdu Biopurify Phytochemicals Ltd. (Chendu, Sichuan, China). Caffeic acid (CA, powder, purity >98%) was from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile and methanol was obtained from sigma company, USA. Solid-phase cartridge was from Waters Chromatography (HLB 6cc, OasisTM, Waters, USA). Deionized water was purified using a mili-Q system (Milipore, Milford, MA, USA). Acetic acid and other chemicals and solvents used were all of analytical grade.

2.2. Drug administration and sample collection

Sprague–Dawley rats (230–250 g) were obtained from academy of military medical sciences (Beijing, China) and housed with unlimited access to food and water except for fasting 12 h before experiment. The rats were maintained on a 12 h light–dark cycle (light on from 8:00 to 20:00 h) at ambient temperature (22–24 °C) and ca. 60% relative humidity. After an oral administration of echinacoside 1.5 g/kg, urine was collected at 6, 12, 24, and 36 h in ice-bath. Feces were also collected at 24 and 36 h. All samples were stored at −80 °C immediately after collection.

2.3. Separation and extraction procedures

Solid phase extraction (SPE) was used to extract the metabolites from the biological samples. All samples were thawed in a water-bath at 37 °C. The 1:2 dilution of urine samples and the 1:10 dilution of feces homogenate samples were added onto the SPE cartridge, which had been previously activated with 4 mL methanol and equilibrated with 4 mL water. After the sample had been absorbed by the cartridge, the cartridge was washed with 2 mL water, and then eluted with 3 mL methanol. The elution was then evaporated under an air stream at 25 °C in a water-bath. The residue was reconstituted in 200 μL of methanol with vortex. After centrifugation at 20,000 × *g* for 10 min, 100 μL of supernatant was drawn and 5 μL was injected into the LC/MS-IT-TOF.

2.4. Instruments and analytical condition

LC experiments were conducted using a Shimadzu (Kyoto, Japan) HPLC system consisting of an LC-20AB binary pump, an SIL-20AC autosampler, a CTO-20AC column oven and an SPD-M20A PDA. Chromatographic separation of analytes was achieved using a 100 mm × 2.0 mm capcell pak C18 MG analytical column (Shiseido, Tokyo, Japan) protected by a Security guard (Phenomenex, USA). The column and autosampler tray temperatures were set at 25 and 4 °C, respectively. The mass detection was carried out using a hybrid Shimadzu ion trap/time-of-flight mass spectrometry (IT-TOF/MS) (Shimadzu, Kyoto, Japan), equipped with an electrospray ionization source. In automatic mode, all ions were firstly accumulated in octopole and then rapidly pulsed into IT for MS^n analysis according to the criteria settings. All ions produced were finally introduced into the TOF instrument for accurate mass determination. Data acquisition and analysis were performed with LC solution 3.0 software (Shimadzu, Kyoto, Japan).

A mobile phase composed of eluent A (0.1% acetic acid in water, v/v) and B (acetonitrile) with a gradient elution was employed for the separation. The mobile phase was programmed as follows: an isocratic elution of 5% B for the first 10 min, followed by a linear gradient elution of 5–10% B from 10 to 20 min, 10% B from 20 to 30 min, 10–30% B from 30 to 50 min and 30–70% B from 50 to 60 min. After holding the solvent composition of 70% B for the next 10 min, the gradient was returned to its starting conditions.

The optimized MS conditions were as follows: negative ion mode; electrospray voltage, −3.5 kV; CDL temperature, 200 °C;

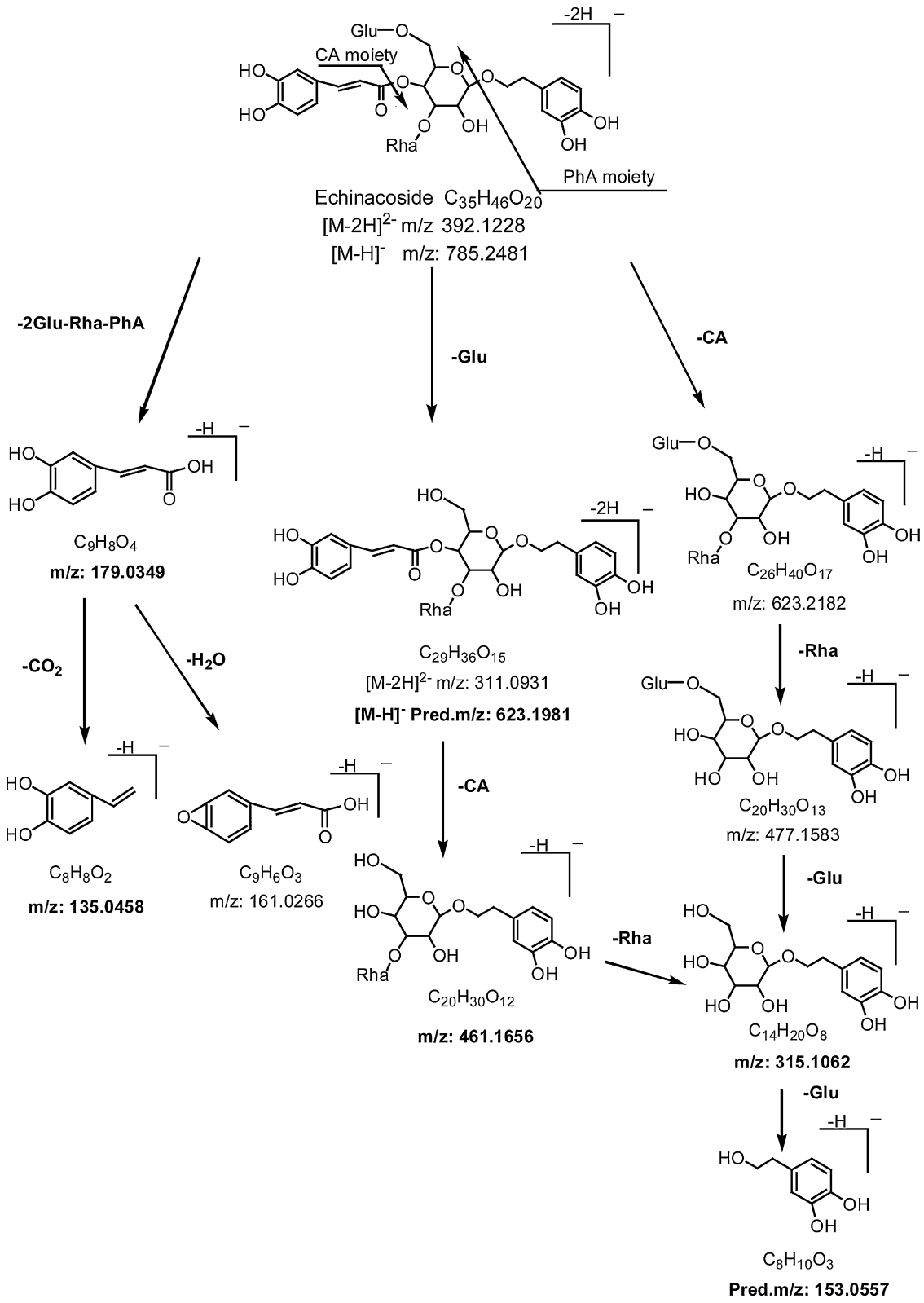

Fig. 1. Proposed fragmentation pathways of echinacoside.

Block Heater temperature, 200 °C; nebulizing gas (N_2), 1.5 L/min; drying gas (N_2) pressure, 0.1 MPa. Mass spectra were acquired in the range of *m/z* 100–1000 for MS^1, 100–1000 for MS^2. The MS^n data were collected in an automatic mode and the software could automatically select precursor ions for MS^n analysis according to criteria settings. Argon was used as the collision gas and the collision energy was set at 150% for MS^2. Prior to data acquisition, the instrument was calibrated with sodium TFA clusters against the entire mass range (*m/z* 50–5000) specified for the instrument.

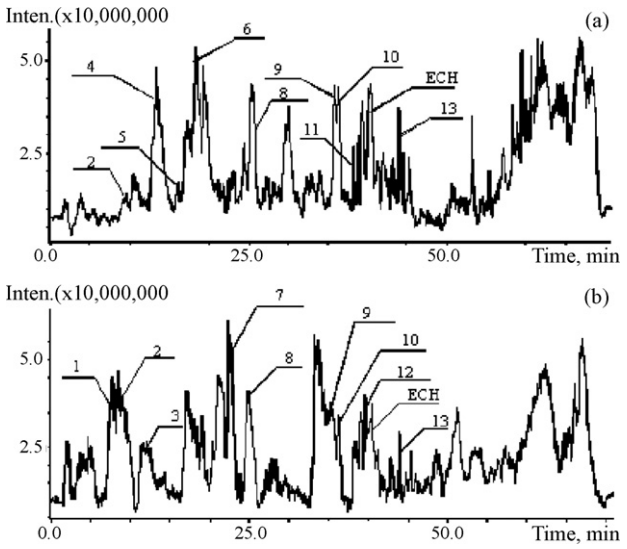

Fig. 2. Total ion chromatograms for the rat urine (a) and feces (b) samples after intragastric administration of echinacoside.

3. Results and discussion

3.1. Fragmentations of echinacoside

Since it has been well proven that the mass fragmentation patterns of metabolites are always similar to the parent compounds, and thus the fragmentation patterns analysis of parent compound is very helpful for the metabolite characterizations, we firstly conducted a detailed fragmentation study of the ECH authentic standard in LC/MS-IT-TOF. To ensure sufficient fragments production, 5 μg/mL of ECH authentic samples prepared in methanol was used for the fragmentation pattern study. Accurate mass measurements of pseudo-molecular and fragment ions of ECH are listed in Table 1. The $[M-2H]^{2-}$ ion at *m/z* 392.1228 ($C_{35}H_{46}O_{20}$) was observed as the predominant pseudo-molecular ion. The product ion at *m/z* 179.0349 ($C_9H_8O_4$) corresponding to CA moiety is one of the major fragments. The fragment at *m/z* 135.0458 ($C_8H_8O_2$) and *m/z* 161.0266 ($C_9H_6O_3$) was formed from the CO_2 and H_2O loss from CA moiety, respectively. The loss of a glucose (Glu) from the double deprotonated pseudo-molecular ion forms a fragment at *m/z* 311.0931 ($[M-2H]^{2-}$, $C_{29}H_{36}O_{15}$), which further losses a CA moiety and produces a fragment at *m/z* 461.1656 ($C_{20}H_{30}O_{12}$). The fragments at *m/z* 623.2182 ($C_{26}H_{40}O_{17}$), *m/z* 477.1583 ($C_{20}H_{30}O_{13}$), and *m/z* 315.1062 ($C_{14}H_{20}O_8$) are produced from the sequential CA, rhamnose (Rha) and Glu moiety loss from the molecular ion, respectively. The proposed fragmentation pattern of ECH is illustrated in Fig. 1.

3.2. Identification of metabolites in rat urine and feces

To ensure the detecting sensitivity of this method, the limit of detection (LOD) and the limit of quantification (LOQ) of quantifying ECH in the rat urine and feces had been validated. The determined LOD (defined as the concentration producing signal to noise ratio over 3) and LOQ (defined as the concentration producing signal to noise ratio over 10 and with the precision variations less than 20%) for quantifying ECH in both biological matrixes was 10 and 25 ng/mL, respectively, indicating that the present LC/MS-IT-TOF based method was sensitive enough to detect and identify the metabolites of ECH at trace level. The representative mass chromatograms for profiling the rat urine and feces samples after intragastric administration of ECH are shown in Fig. 2. The peaks corresponding to potential metabolites were determined from the

Table 2
Retention time (t_R) and accurate mass measurements of echinacoside and all metabolites in rat urine and feces samples.

Peak No.	t_R (min)	Pseudo-molecular ion *m/z* (formula, mDa[a], ppm[b])	Fragment ion *m/z* (mDa, ppm)	Identification
1	8.0	153.0561 ($C_8H_{10}O_3$, 0.4, 2.61)	123.0473 (2.1, 17.07)	3,4-Dihydroxyphenethyl alcohol
2	10.0	233.0119 ($C_8H_{10}O_6S$, −0.6, 2.57)	153.0577 (0.00, 0.00), 123.0467 (1.5, 12.19)	3,4-Dihydroxyphenethyl alcohol sulfate conjugate
3	12.0	461.1668 ($C_{20}H_{30}O_{12}$, 0.6, 1.30)	315.1054 (−3.1, −9.84), 161.0460 (0.5, 3.10)	Decaffeoyl verbascoside
4	12.7	405.1399 ($C_{17}H_{26}O_{11}$, −0.3, −0.74)	345.1187 (−0.4, −1.16), 165.0588 (3.1, 18.78)	Hydroxyl methyl 3,4-dihydroxyphenethyl glycoside
5	16.0	258.9923 ($C_9H_8O_7S$, 0.3, 1.16)	179.0381 (3.1, 17.32)	Caffeic acid sulfate conjugate
6	18.0	389.1457 ($C_{17}H_{26}O_{10}$, 0.4, 1.03)	329.1225 (0.4, 1.03), 167.0740 (−1.7, 5.17), 123.0472 (2.6, 15.56)	Methyl 3,4-dihydroxyphenethyl glycoside
7	23.0	179.0359 ($C_9H_8O_4$, 0.9, 5.03)	135.0474 (2.2, 16.29)	Caffeic acid
8	26.0	432.0996[c] ($C_{35}H_{46}O_{23}S$, 0.8, 1.85)	392.1205[c] (−1.3, 3.32), 179.0368 (1.8, 10.05), 703.1769 (0.8, 1.14), 215.0038 (1.8, 8.37)	Echinacoside sulfate conjugate I
9	35.8	432.1006[c] ($C_{35}H_{46}O_{23}S$, 0.2, 0.46)	703.1692 (−6.9, −9.82), 161.0253 (0.9, 5.59),	Echinacoside sulfate conjugate II
10	36.3	400.1196[c] ($C_{35}H_{46}O_{21}$, 0.3, 0.75)	391.112[c] (−1.1, −2.81), 179.0363 (−1.3, −7.26), 135.0479 (2.7, 19.99)	Hydroxyl echinacoside
11	38.8	480.1386[c] ($C_{41}H_{54}O_{26}$, 1.5, 3.12)	623.2241 (4.8, 7.70), 785.2481 (−2.9, 3.69), 392.1218[c] (0.0, 0.00)	Echinacoside glucuronide conjugate
12	39.7	432.1017[c] ($C_{35}H_{46}O_{23}S$, 1.3, 3.01)	258.9908 (1.0, 3.86)	Echinacoside sulfate conjugate III
13	44.2	623.2024 ($C_{29}H_{36}O_{15}$, 4.3, 6.90)	461.1688 (2.6, 5.64), 315.1116 (3.1, 9.48)	Verbascoside

[a] Milli-Dalton, differences between the measured and predicted values.
[b] Differences between the measured and predicted values.
[c] $[M-2H]^{2-}$ ions in negative scan mode, while others were $[M-H]^-$ ions.

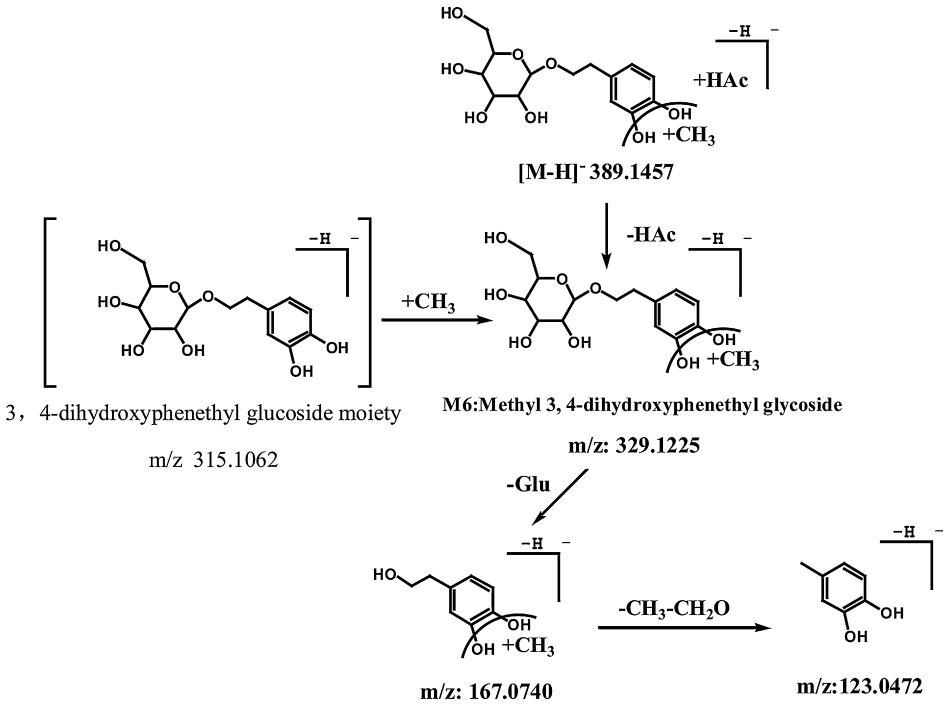

Fig. 3. Proposed fragmentation mechanism of M6.

automated mass chromatogram comparisons between the blank samples and the drug treated samples. The retention time, accurate mass data of the parent and fragment ions for the parent compound and all metabolites are collected in Table 2. A total of 13 metabolites were found from the rat urine and feces samples. The metabolites identification strategy and results were elucidated in detail as follows.

3.2.1. Identification of degradation products via direct fragment comparisons

The chemical structure of ECH is composed of three chemical moieties including CA, phenylethanoid aglycone (PhA, 3,4-dihydroxyphenethyl alcohol, hydroxytyrosol), and sugar. According to the current knowledge of drug metabolism, ECH are readily subject to hydrolysis under the biological conditions, especially cat-

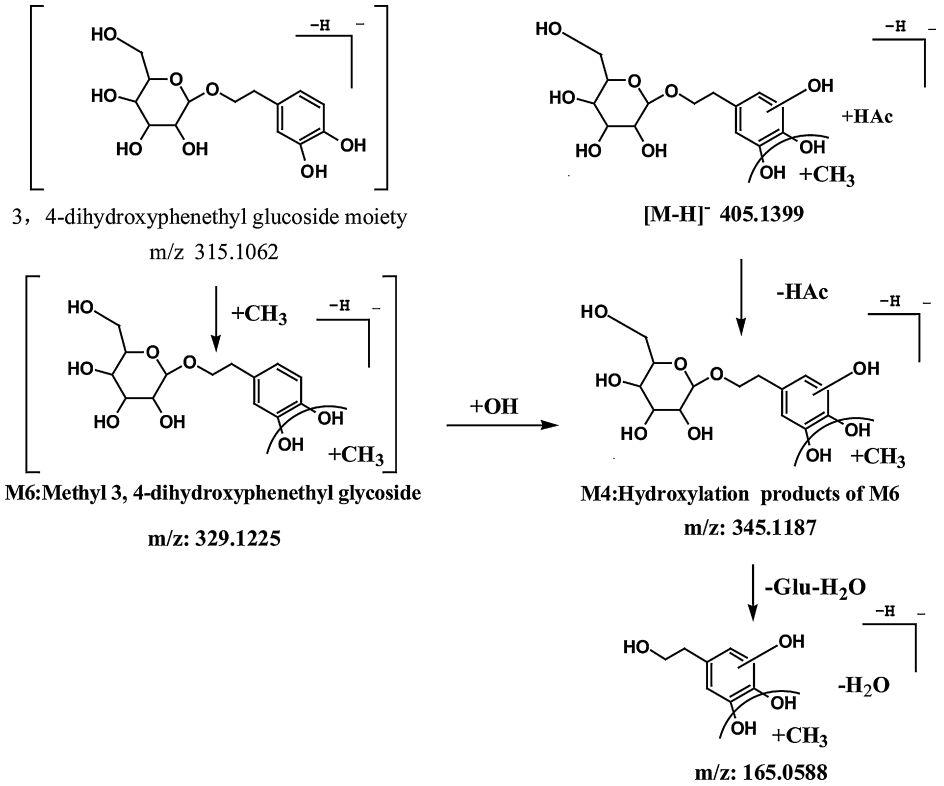

Fig. 4. Proposed fragmentation mechanism of M4.

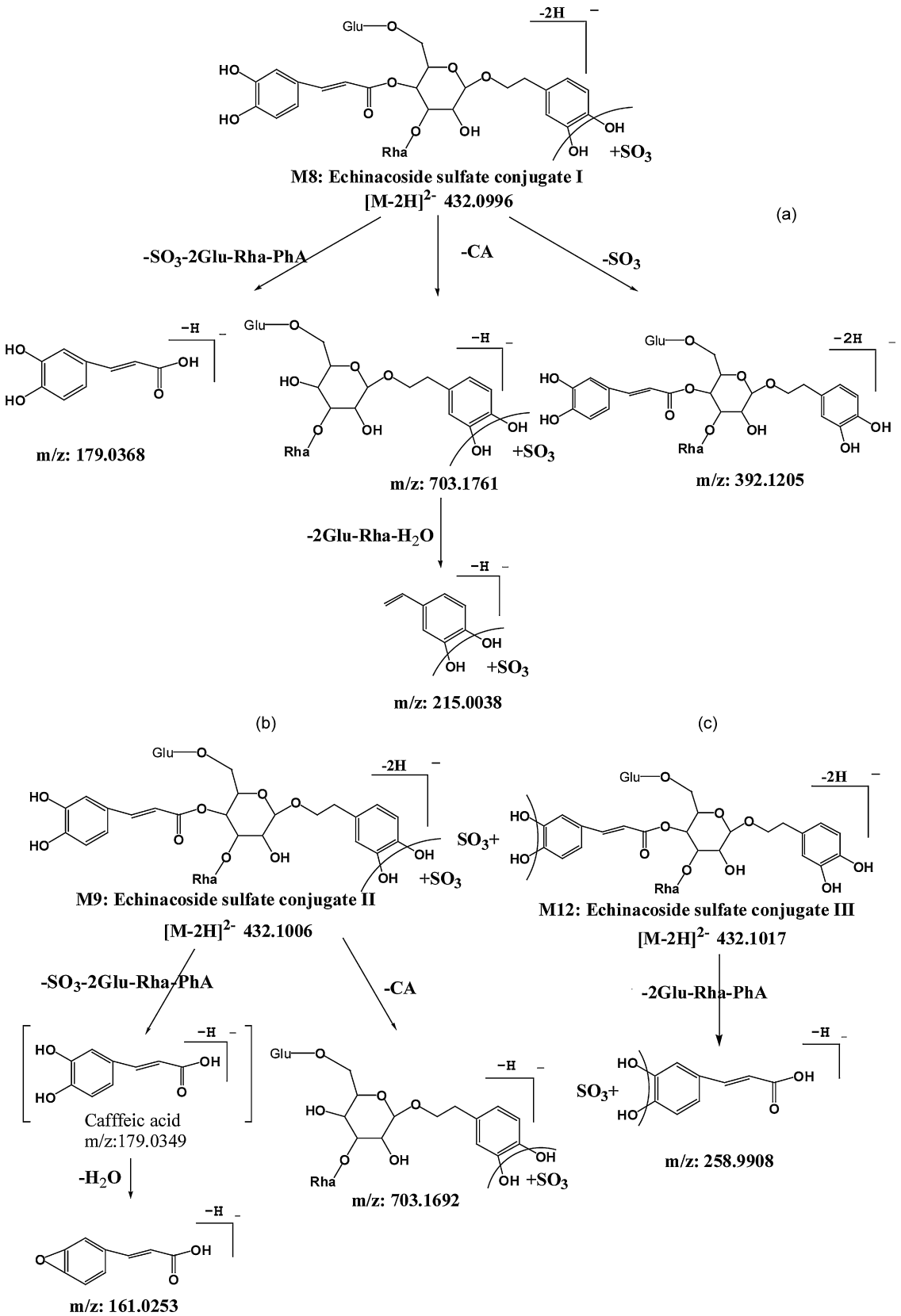

Fig. 5. Proposed fragmentation mechanism of M8 (a), M9 (b), M12 (c).

alyzed by the intestinal microflora. Therefore, we proposed that ECH may produce several degradation products in the rat gastrointestinal tract. Recently, García-Reyes et al. [28] proposed a "fragmentation–degradation" correlation strategy to identify the degradation products based on the fact that the compounds degradation pathways are always similar to the fragmentation patterns in the mass CID conditions. For this consideration, we applied such a strategy to search for the potential degradation prod-

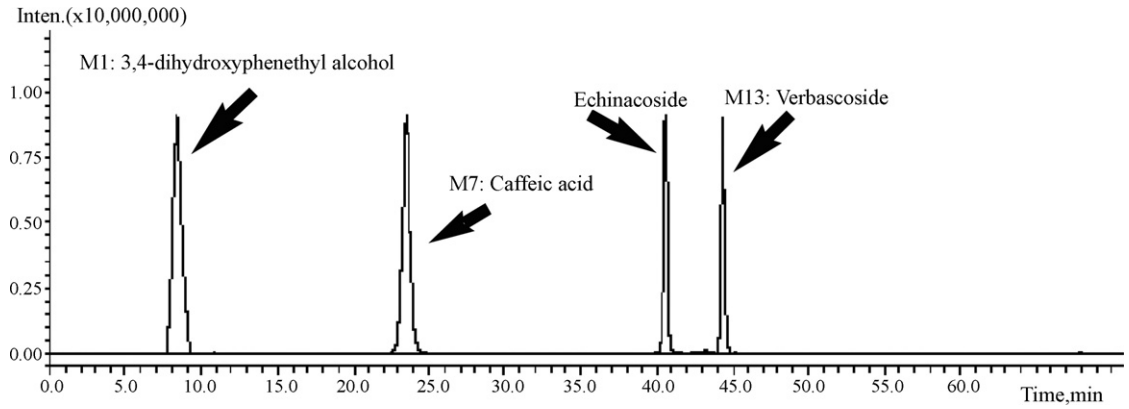

Fig. 6. Extracted ion chromatogram of 3,4-dihydroxyphenethyl alcohol, caffeic acid and verbascoside standards.

ucts of ECH using the determined fragments of ECH authentic standard.

The determined and predicted fragment ions of ECH were searched against the mass chromatograms of rat urine and feces samples. A total of four metabolites have been found to characterize the pseudo-molecular ions with same *m*/*z* and calculated elemental compositions to that of the ECH fragments. M7, eluted at 23.0 min, is characterized with the pseudo-molecular ion at *m*/*z* 179.0359 ($C_9H_8O_4$), which is actually identical to the ECH fragment ion at *m*/*z* 179.0349 ($C_9H_8O_4$, caffeic acid moiety). The major fragment ion determined at *m*/*z* 135.0474 ($C_8H_8O_2$) for M7 is also same to that of the caffeic acid moiety. Therefore, M7 is identified as a degradation product of echinacoside, caffeic acid. M13, eluted at 44.2 min, characterized with the deprotonated molecule ion at *m*/*z* 623.2024 ($C_{29}H_{36}O_{15}$) which is really close to the de-glucose fragment ion (*m*/*z* 623.1981) of ECH, is proposed to be the de-glucose product of ECH named as verbascoside. The major fragment ions of M13 (*m*/*z* 461.1688, *m*/*z* 315.1116) are also same to that from the verbascoside moiety. Based on this rule, M3 (Rt, 12.0 min; *m*/*z* 461.1668) and M1 (Rt, 8.0 min; *m*/*z* 153.0561) is identified as the decaffeoyl product of verbascoside and 3,4-dihydroxyphenethyl alcohol, respectively.

3.2.2. Phase I and phase II metabolites of degradation products

The degradation products can further undergo phase I and/or phase II metabolism. Since such metabolites are always produced from multiple metabolic reactions from the parent compound, it is relatively difficult to characterize their structures. In this study, we proposed a strategy for rapidly characterizing the phase I and phase II metabolites of the degradation products. Firstly, the pseudo-molecular ions of such metabolites were compared with those of the determined degradation products. With the benefit of accurate mass measurements by TOF, it is feasible to obtain the elemental composition differences between the target metabolites and their precursors (degradation products). Subsequently, the reasonable elemental composition difference could be determined based on the current understanding of the metabolic routes. Finally, the fragmentation patterns comparisons would help to confirm the characterizations. The observed quasi-molecular ion for M5, eluted at 16.0 min, was at *m*/*z* 258.9923, which was 80 Da over that of the degradation product M7, caffeic acid. The major fragment ion of M5 at *m*/*z* 179.0381 ($C_9H_8O_4$) confirms that M5 is caffeic acid sulfate conjugate. Similarly, M2 (Rt, 10.0 min) characterized with the quasi-molecular ion at *m*/*z* 233.0119 was readily identified as the sulfate conjugate of M1, 3,4-dihydroxyphenethyl alcohol.

However, some degradation products of the parent compound might be immediately and completely metabolized and thus could not be detected. The identification of such kind of metabolites when their precursors were not detected would be more difficult. In view that the degradation modes are always similar to the mass fragmentation patterns, we proposed here that the multiple crossover comparisons of the quasi-molecular ions with the fragments of the parent compound would help for characterizing such metabolites. As is in this study, M6 has been rapidly characterized following such a strategy. As shown in Fig. 3, M6 eluted at 18.0 min produced a predominant quasi-molecular ion at *m*/*z* 389.1457 ($C_{17}H_{26}O_{10}$), which was presumed to be an acetic adductive ion as evidenced from its elemental composition difference ($C_2H_4O_2$) from the deprotonated ion at *m*/*z* 329.1225 ($C_{15}H_{22}O_8$). Comparing the deprotonated molecular ion of M6 with that of the defined degradation products leaded to none of reasonable results, suggesting that M6 was not a subsequent metabolite of the detected degradation products. When matching the quasi-molecular ion of M6 with the fragments of parent compound, a 14 Da difference corresponding to a methyl group has been observed from a fragment at *m*/*z* 315.1062 ($C_{14}H_{20}O_8$), which was the 3,4-dihydroxyphenethyl glucoside moiety. Therefore, M6 was proposed to be a methyl 3,4-dihydroxyphenethyl glycoside. The fragmentation pattern of M6 supported such a characterization. The loss of a Glu from the deprotonated molecular ion produced a fragment ion at *m*/*z* 167.0740 ($C_9H_{12}O_3$), which further produced a fragment ion at *m*/*z* 123.0472 ($C_7H_8O_2$) by the CH_3 and CH_2O dissociation.

M4, eluted at 12.7 min, showed a major quasi-molecule ion at *m*/*z* 405.1399 ($C_{17}H_{26}O_{11}$) which was also presumed to be an acetic acid adduct ion since it also produced a deprotonated molecular ion at *m*/*z* 345.1187. The predicted elemental composition of $C_{15}H_{22}O_9$ for M4 is an oxygen atom more than that of the M6 and a CH_2O more than the fragment at *m*/*z* 315.1062 ($C_{14}H_{20}O_8$). Therefore, the M4 was tentatively presumed to be the subsequent hydroxylation products of M6. As shown in Fig. 4 the major fragment ion at *m*/*z* 165.0588 ($C_9H_{10}O_3$) presumed to be produced via losing a Glu moiety and a H_2O group from the deprotonated molecular ion of M4 supported such a characterization.

3.2.3. Phase I and phase II metabolites of echinacoside

The direct phase I and phase II metabolites of a compound are relatively easy to be characterized based on the fragmentation comparisons with the parent compound. M8, M9 and M12, characterized with similar quasi-molecular ion ($[M-2H]^{2-}$) mass at *m*/*z* 432.0996, *m*/*z* 432.1006 and *m*/*z* 432.1017 and same predicted elemental composition of $C_{35}H_{46}O_{23}S$, suggested that they were the sulfate conjugation products at different hydroxyl groups. The further fragmentation patterns and retention behavior analysis helped us to determine the sulfate conjugating positions for M8, M9 and M12. The fragment ion at *m*/*z* 703.1769 ($C_{26}H_{40}O_{20}S$) was a CA moiety loss from the molecular ion, which suggested that the sulfate conjugation position was not at the CA moiety for M8. In addition,

Table 3
Comparison of retention time (t_R) and accurate mass data for the metabolites proposed and their respective authentic standards.

Peak No.	Compound name	Metabolites			Standards		
		t_R (min)	Pseudo-molecular ion m/z (ppm)	Fragment ion m/z (ppm)	t_R (min)	Pseudo-molecular ion m/z (ppm)	Fragment ion m/z (ppm)
1	3,4-Dihydroxyphenethyl alcohol	8.0	153.0561 (2.61)	123.0473 (17.07)	8.3	153.0563 (3.92)	123.0462 (8.13)
7	Caffeic acid	23.0	179.0359 (5.03)	135.0474 (16.29)	23.4	179.0349 (−0.56)	135.0458 (4.44)
13	Verbascoside	44.2	623.2024 (6.90)	461.1688 (5.64), 315.1116 (9.48)	44.4	623.1999 (2.89)	461.1643 (−4.77), 315.1044 (5.40)

the fragment ion at m/z 215.0038 ($C_8H_8O_5S$) presumed to be a H_2O loss from 3,4-dihydroxyphenethyl alcohol sulfate conjugate (m/z 233.0119, $C_8H_{10}O_6S$), confirmed that the site of sulfate conjugant of M8 was at PhA moiety. For M9, the major fragment ion at m/z 703.1692 ($C_{26}H_{40}O_{20}S$) was formed through a CA moiety loss from the quasi-molecular ion, suggesting that the sulfate was conjugated with the hydroxyl group at PhA moiety. The sole fragment ion at m/z 258.9908 ($C_9H_8O_7S$) for M12 which was proposed to be the caffeic acid sulfate conjugate (m/z 258.9923, $C_9H_8O_7S$) supported that the sulfate conjugation was formed at the CA moiety. The fragmentation patterns of M8, M9 and M12 are illustrated in Fig. 5 separately.

M10, eluted at 36.3 min, showed the predominant quasi-molecular ion $[M-2H]^{2-}$ at m/z 400.1192 ($C_{35}H_{46}O_{21}$) that is an oxygen atom more than the parent compound ECH, suggesting that it was a hydroxylation product of ECH. The major fragment ion at m/z 391.1129 ($C_{35}H_{44}O_{20}$) was a H_2O loss from the quasi-molecular ion. The fragment ions found at m/z 179.0363 ($C_9H_8O_4$) and m/z 135.0479 ($C_8H_8O_2$) were same to that of the parent compound ECH, suggested that the hydroxylation position was not on the caffeic acid moiety. In addition, in view that the H_2O loss fragmentation for M10 has not been observed from the parent compound, we proposed that the hydroxylation was more likely to occur on the PhA moiety to form a benzenetriol structure.

M11, eluted at 38.8 min, was characterized with the major qausi-molecular ion $[M-2H]^{2-}$ at m/z 480.1386 ($C_{41}H_{54}O_{26}$). The double and mono-deprotonated fragment at m/z 392.1218 ($C_{35}H_{46}O_{20}$) and m/z 785.2481 ($C_{35}H_{46}O_{20}$) that was formed through a glucuronic acid (176 Da, $C_6H_8O_6$) loss from the parent ion of M11, supporting that M11 was a glucuronic conjugate of ECH. Due to the insufficient fragmentation information, we failed to determine the conjugation position.

3.2.4. *Metabolites identification verified by authentic standards*

To further confirm the metabolite identifications, three major metabolites M1, M7 and M13 that were proposed as 3,4-dihydroxyphenethyl alcohol, caffeic acid and verbascoside, respectively, were verified by using authentic standards. The three compounds were prepared in methanol at 5 μg/mL of each as a mixture and injected into the instrument which was run under same conditions as that used for the metabolites identification. The obtained extracted ion chromatogram is shown in Fig. 6 and the retention time and mass spectra data are summarized in Table 3. The retention times and accurate mass data comparisons showed that the metabolites proposed from the real world biological samples (rat urine and feces) matched well with their respective authentic standards, further supporting the original structure characterizations.

4. Conclusion

This study proposed a systematic approach for rapid metabolites identification of such compounds as ECH undergoing multiple and unpredictable metabolism from biological matrix, based on the LC/MS-IT-TOF analysis. Firstly, the degradation products of ECH were characterized via degradation and fragmentation relationship analysis. Based on the fragmentation patterns analysis of ECH, four degradation products were rapidly identified. Secondly, the further metabolites of the degradation products were characterized by the elemental composition differences analysis of the quasi-molecular ions between the metabolites and their precursors. Finally, the direct phase I and phase II metabolites of the parent compound were readily characterized by the quasi-molecular ion mass difference analysis. With the benefit from the sufficient fragments information, the metabolic positions can be determined in most cases. Using this approach, a total of 13 metabolites of ECH has

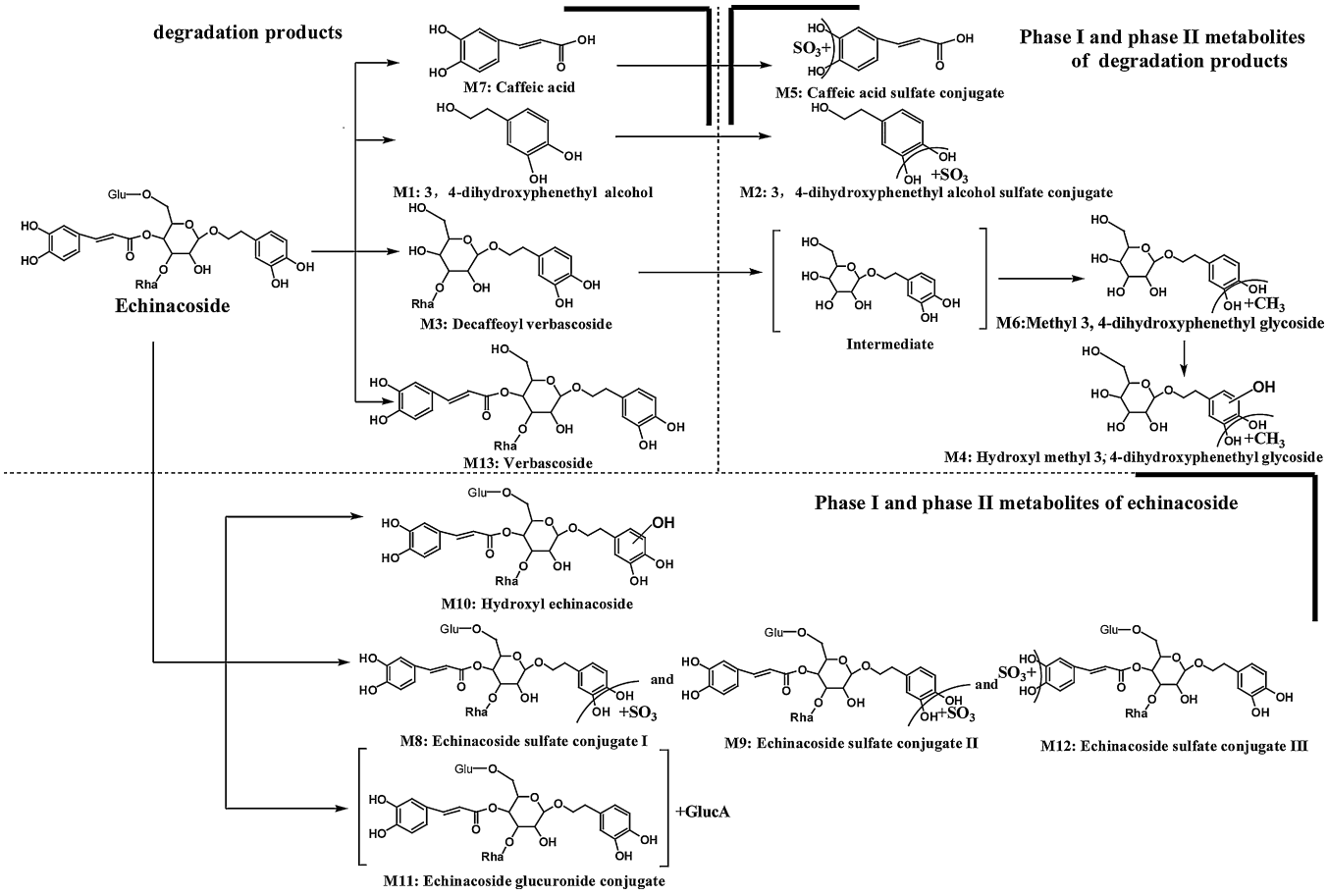

Fig. 7. Proposed metabolic pathways of echinacoside.

been identified from the rat urine and feces after an intragastric dosage. Fig. 7 depicts the identified metabolic pathways for ECH, including degradation products, and glucuronic acid, sulfate, and methyl conjugation of both parent compound and the degradation products.

In summary, the present study provides a more comprehensive map of ECH metabolism than that from the previously published results [15], where only eight phase II metabolites of the parent compound ECH had been identified. In addition, this study presents a feasible and generally applicable strategy for the complicated metabolites identification of natural products.

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References

[1] I. Saracoglu, M. Inoue, I. Calis, Y. Oglihara, Biol. Pharm. Bull. 18 (1995) 1396.
[2] M. Pennacchio, E. Alexander, Y.M. Syah, Euro. J. Pharmacol. 305 (1996) 169.
[3] Q.B. Xiong, K. Hase, Y. Tezuka, T. Tani, Planta Med. 64 (1998) 120.
[4] Q.B. Xiong, S. Kadota, T. Tani, T. Namba, Biol. Pharm. Bull. 19 (1996) 1580.
[5] M. Deng, J.Y. Zhao, P.F. Tu, Y. Jiang, Euro. J. Pharmacol. 505 (2004) 11.
[6] P.F. Tu, B. Wang, T. Deyama, Z.G. Zhang, Acta Pharm. Sin. 32 (1997) 294.
[7] N.B. Perry, E.J. Burgess, V.L. Glennie, J. Agric. Food Chem. 49 (2001) 1702.
[8] R. Cervellati, C. Renzulli, M.C. Guerra, E. Speroni, J. Agric. Food Chem. 50 (2002) 7504.
[9] C. Hu, D.D. Kitts, J. Agric. Food Chem. 48 (2000) 1466.
[10] F. Pellati, S. Benvenuti, L. Magro, M. Melegari, J. Pharm. Biomed. Anal. 35 (2004) 289.
[11] K.A. Koo, S.H. Sung, J.H. Park, S.H. Kim, Planta Med. 71 (2005) 778.
[12] E. Speroni, P. Covoni, S. Guizzardi, C. Renzulli, J. Ethnopharmacol. 79 (2002) 265.
[13] A. Matthias, R.S. Addison, K.G. Penman, R.G. Dickinson, Life Sci. 77 (2005) 2018.
[14] C.Q. Jia, H.M. Shi, X.M. Wu, J. Chromatogr. B 844 (2006) 308.
[15] C.Q. Jia, H.M. Shi, W. Jin, Drug Metab. Dispos. 37 (2009) 431.
[16] R.J. Steen, I. Bobeldijk, U.A. Brinkman, J. Chromatogr. A 915 (2001) 129.
[17] S. Lacorte, A.R. Fernandez-Alba, Mass Spectrom. Rev. 25 (2006) 866.
[18] I. Ferrer, E.M. Thurman, J. Chromatogr. A 1175 (2007) 24.
[19] E.M. Thurman, I. Ferrer, A.R. Fermandez-Alba, J. Chromatogr. A 1067 (2005) 127.
[20] E.M. Thurman, I. Ferrer, J.A. Zweigenbaum, J. Chromatogr. A 1082 (2005) 71.
[21] I. Ferrer, C.E. Heine, E.M. Thurman, Anal. Chem. 76 (2004) 1437.
[22] H.P. Hao, N. Cui, G.J. Wang, B.R. Xiang, Anal. Chem. 80 (2008) 8187.
[23] C.N. Zheng, H.P. Hao, X. Wang, G.J. Wang, J. Mass Spectrom. 44 (2009) 230.
[24] F.X. Zhang, Z.H. Jia, P. Gao, H.W. Kong, X. Li, J. Chen, Q. Yang, P.Y. Yin, J.S. Wang, X. Liu, F.M. Li, Y.L. Wu, G.W. Xu, Talanta 79 (2009) 836.
[25] H.Y. Zhang, X. Cheng, P. Hu, Q.L. Liang, X.P. Liang, Y.M. Wang, G.A. Luo, Talanta 79 (2009) 254.
[26] J. Zhang, Y. Jin, J. Dong, Y. Xiao, J. Feng, X. Xue, X. Zhang, X. Liang, Talanta 78 (2009) 513.
[27] R. Guo, Q. Zhou, Y. Cai, G. Jiang, Talanta 75 (2008) 1394.
[28] J.F. García-Reyes, A. Molina-Díaz, A.R. Fernandez-Alba, Anal. Chem. 79 (2007) 307.