Metabolism of Echinacoside, a Good Antioxidant, in Rats: Isolation and Identification of Its Biliary Metabolites

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ABSTRACT:

Echinacoside (ECH) is one of the major active phenylethanoid glycosides (PEGs) in famous traditional Chinese medicine, Herba Cistanches. Although it has various bioactivities, such as antioxidation, neuroprotection, and hepatoprotection, knowledge about its metabolic fate is scant. In the present study, eight phase II metabolites, 3,4‘‘’’-O-dimethyl-ECH-3’’’’-O-β-D-glucuronide (M1); 4,4‘‘’’-O-dimethyl-ECH-3’’’’-O-β-D-glucuronide (M2); 3,4‘‘’’-O-dimethyl-ECH-4-O-sulfate ester (M3); 4,4‘‘’’-O-dimethyl-ECH-3-O-sulfate ester (M4); 3,3’’’’-O-dimethyl-ECH (M5); 3,4‘‘’’-O-dimethyl-ECH (M6); 4,3‘‘’’-O-dimethyl-ECH (M7); and 4,4‘‘’’-O-dimethyl-ECH (M8), were isolated from rat bile sample after intravenous administration of ECH and identified by mass spectra and NMR spectroscopy, including 1H NMR, 13C NMR, nuclear Overhauser effect difference spectroscopy, and two-dimensional NMR (heteronuclear single quantum correlation, heteronuclear multiple-bond correlation spectroscopy, gradient-selected correlation spectroscopy, and nuclear Overhauser effect spectroscopy). Among them, M5 to M8 were O-di-methylated conjugates; M1 and M2 and M3 and M4 were O-dimethyl glucuronides and O-dimethyl sulfates, respectively. In the three types of metabolites of rat, the major metabolites were the methyl ethers and the glucuronsides, whereas the sulfates were minor. The regioselectivity of conjugation for ECH and metabolic pathway of ECH were proposed, which gave insight into the mechanism of ECH for its bioactivities in vivo.

Phenylethanoid glycosides (PEGs), as a type of polyphenolic compounds, are widely distributed in different plant families and possess various pharmacological effects, such as antioxidation, anti-inflammation, antioxygen, immunomodulation. Echinacoside (ECH; [2R,3R,4R,5R,6R]-6-2-[3,4-dihydroxyphenylethoxy]-5-hydroxy-2-[[2R,3R,4S,5S,6R]-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl][oxymethyl]-4-[[2S,3S,4R,5R,6S]-3,4,5-trihydroxy-6-methyloxan-2-yl]oxano-3-yl][E]-3-(3,4-dihydroxyphenyl)prop-2-enoate) is one of the representative and major PEGs in Herba Cistanches (Tu et al., 1997), which is an important traditional Chinese medicine used for the treatment of kidney deficiency and neurodegenerative diseases. ECH is also the main phenolic component in Echinaea angustifolia and Echinaea pallida roots (Perry et al., 2001), which are widely used in Europe, North America, and Australia for their immunostimulating activities. As a natural polyphenolic compound, ECH showed various bioactivities, such as antioxidative (Li et al., 1992; Xiong et al., 1996; Hu and Kitts, 2000; Cervellati et al., 2002), neuroprotective (Den et al., 2004; Koo et al., 2005; Chen et al., 2007; Geng et al., 2007), nitric oxide radical-scavenging (Xiong et al., 2000), and antihepatotoxic activities (Houghton and Hikino, 1989; Wu et al., 2007).

Although ECH exhibited diverse bioactivities in in vivo and in vitro assays, only a few reports dealing with the metabolism and disposition of ECH are available. In our previous study on the pharmacokinetics and bioavailability of ECH in rat, the distribution and elimination of ECH were extremely fast (τ1/2α, 12.4 min; τ1/2β, 41.0 min), clearance was 0.0001 mg/kg/min/ng/ml after i.v. administration (5 mg/kg), and the bioavailability of ECH was only 0.83% (Jia et al., 2006). The very low bioavailability of ECH was consistent with the results of Matthias et al. (2004), in which ECH permeated poorly through the Caco-2 monolayers. Moreover, our pharmacokinetic data suggested that ECH was mostly metabolized after dosing. Therefore, we hypothesized that, in addition to the parent compound, some metabolic products of ECH may show biological activities, thereby contributing to the pharmacological efficacy of ECH in vivo. It was necessary to...
make the metabolic pathway of ECH clear to provide insight into the mechanism(s) through which it exerts its beneficial physiological effects. As a follow-up study, we isolated and identified eight metabolites of ECH from rat bile.

Materials and Methods

Materials and Chemicals. Echinacoside was separated and purified from an ethanol extract of *Cistanche tubulosa* (Schenk) B. Wright by our laboratory, and its structure was confirmed by UV, IR, MS, and NMR spectroscopy. The purity was 96.4% as determined by HPLC. HPLC grade acetonitrile was obtained from Caledon Laboratories Ltd. (Georgetown, ON, Canada). HPLC grade methanol was obtained from Tianjin Concord International Trade Co., Ltd. (Tianjin, China). Trifluoroacetic acid was analytical grade. Water was HPLC grade acetonitrile and its structure was confirmed by UV, IR, MS, and NMR spectroscopy. The purity was 96.4% as determined by HPLC analyses.

Animals. Male Sprague-Dawley rats (200–310 g) were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, People’s Republic of China). They were kept in an environmentally controlled animal room (20 ± 2°C, 60 ± 5% humidity; 12-h dark/light cycle) for 3 days before starting the experiments. Rats were fed standard laboratory food with water ad libitum and fasted overnight before the test.

Bile Collection. Under light anesthesia with diethyl ether, bile fistulas in 12 rats were cannulated with polyethylene-5 tubing for collection of bile. The bile was collected into successive vials on ice at 2-h intervals for 8 h after dosing and at 8- to 12-, 12- to 24-, 24- to 30-h intervals thereafter. The rats were allowed to recover from anesthesia before receiving a 5 mg/kg i.v. dose of ECH

Altogether, we isolated and identified eight metabolites of ECH from rat bile. To give three fractions (Fr. 1–3), with *R*$_t$ values of 0.77, 0.15, and 0.45 on polyamide TLC (MeOH:H$_2$O/HCOOH = 15:35:0.5, freshly prepared; blue spots after spraying with FeCl$_3$-K$_3$[Fe(CN)$_6$]), respectively. Fr. 1 to 3 were subjected to preparative HPLC [MeCN-0.05% trifluoroacetic acid (16:84)], respectively. Fr. 1 yielded M1 (25 mg; *t*$_R$ = 19 min) and M2 (23 mg; *t*$_R$ = 27 min); Fr. 2 yielded M3 (15 mg; *t*$_R$ = 12 min) and M4 (15 mg; *t*$_R$ = 22 min); and Fr. 3 yielded M5 (18 mg; *t*$_R$ = 42 min), M6 (63 mg; *t*$_R$ = 48 min), M7 (12 mg; *t*$_R$ = 56 min), and M8 (21 mg, *t*$_R$ = 66 min). The purity of each metabolite was 91, 90, 96, 95, 96, 93, 96, and 95%, respectively, based on HPLC analyses.

**Results**

Isolation and Structure Elucidation of the Eight Metabolites. HPLC chromatography of the bile samples from rats administrated ECH intravenously or orally revealed eight new large peaks, named M1 to M8 (Fig. 1B). The retention time of the eight metabolites was longer than that of ECH, which was hardly detected. The retention time of ECH was approximately 9.7 min.

By the above-mentioned methods, eight metabolites were obtained: M1 (25 mg), M2 (23 mg), M3 (15 mg), M4 (15 mg), M5 (18 mg), M6 (63 mg), M7 (12 mg), and M8 (21 mg). MS, IR, ID, and 2D spectra of M1–M8 in CD$_3$OD is detailed in the Supplemental Data. Their structures are shown in Fig. 2.

Metabolite M6 was obtained as an amorphous light yellowish powder. Its HR-ESI-MS exhibited a pseudomolecular ion [M – H]$^-$ at *m/z* 813.2823, (calc. for C$_{37}$H$_{49}$O$_{20}$; 813.2796), compatible with the molecular formula C$_{37}$H$_{49}$O$_{20}$. The IR spectrum showed absorption bands typical of hydroxyls (3418 cm$^{-1}$), α,β-unsaturated ester (1678; 1631 cm$^{-1}$), and aromatic rings (1610; 1516 cm$^{-1}$). The 1H NMR of M6 (Table 1) exhibited characteristic proton signals of a trans-(3,4-di-hydroxy-phenyl)-acrylic acid (E-caffeoyl) group [three aromatic protons at 6.710 (d, *J* = 2.0 Hz), 7.066 (dd, *J* = 8.5, 2.0 Hz), and 6.922 (d, *J* = 8.5 Hz) as an ABX system; two trans-olefinic protons as an AX system at δ 7.63 (d, *J* = 16.0 Hz) and 6.34 (d, *J* = 16.0 Hz)]; a set of protons on the 1,3,4-trisubstituted benzene ring [δ 6.85 (d, *J* = 2.0 Hz), 6.72 (d, *J* = 8.0 Hz), and 6.68 (d, *J* = 8.0, 2.0 Hz); a triplet at δ 2.85 due to a β-methene; and two nonequivalent protons at δ 4.05 and 3.75 of the side chain of the aglycone moiety. Comparison of the 1H and 13C NMR data (Tables 1 and 2) of M6 with those of ECH suggested that the structure of M6 is closely related to that of ECH, except for two additional methoxyl groups. The location of the two methoxyl groups at C-3 and C-4 was inferred by the NOESY spectrum (Warashina et al., 1992). In the NOESY spectrum, one methoxyl signal at 3.86 showed a NOE effect with H-5 (δ 6.85), one of the aromatic protons in the aglycone moiety, and another methoxyl signal at δ 3.86 showed a NOE effect with H-5” (δ 6.92) in the ester moiety. On the basis of the spectral evidence, mainly 2D-NMR (HMBC and HSQC), the structure of M6 was elucidated as 2-(3-methoxy-4-hydroxyphenyl)-ethyl O-α-l-rhamnopyranosyl-(1→3)-[β-d-glucopyranosyl-(1→6)]-(4-O-E-isofuroyl-(1→β)-d-glucopyranoside.

Metabolite M5 was also obtained as an amorphous light yellowish powder. The positive ESI-MS showed two pseudomolecular ion peaks at *m/z* 832.2 [M + NH$_4$]$^+$ and 837.2 [M + Na]$^+$, and the negative ESI-MS gave a pseudomolecular ion peak at *m/z* 813.1 [M – H]$^-$, which corresponds to the molecular formula C$_{37}$H$_{49}$O$_{20}$ by combining the 1H and 13C NMR data (Tables 1 and 2). On the basis of spectral evidence, mainly 2D-NMR (HMBC and HSQC), the structure of M5, an isomer of M6, was elucidated as cistanoside B, which was isolated previously from *Cistanche salsa* as a natural product (Kobayashi et al., 1984).

Metabolite M7 was also obtained as an amorphous light yellowish powder. The positive ESI-MS showed two pseudomolecular ion peaks
at m/z 832.1 [M + NH₄]⁺ and 837.1 [M + Na]⁺, and the negative ESI-MS gave a pseudomolecular ion peak at m/z 813.2 [M – H]⁻, which corresponds to the molecular formula CₙH₂₅O₂₀ by combining the ¹H and ¹³C NMR data (Tables 1 and 2). By careful analysis of 2D-NMR (HMBC and HSQC) spectral data, the structure of M₇, an isomer of M₆, was deduced as 2-(3-hydroxy-4-methoxyphenyl)-ethyl O-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-(4-O-E-feruloyl)-β-D-glucopyranoside, which was obtained previously from Verbascum thapsus as a natural product (Warashina et al., 1992).

Metabolite M₈ was also obtained as an amorphous light yellowish powder. The positive ESI-MS showed a pseudomolecular ion peak at m/z 813.2 [M – H]⁻, which corresponds to the molecular formula CₙH₂₅O₂₀ by combining the ¹H and ¹³C NMR data (Tables 1 and 2). By careful analysis of 2D-NMR (HMBC and HSQC) spectral data, the structure of M₈, an isomer of M₆, was deduced as 2-(3-hydroxy-4-methoxyphenyl)-ethyl O-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-(4-O-E-feruloyl)-β-D-glucopyranoside, which has been isolated from Verbascum sinaiticum as a natural product (Elgindi and Mabry, 2000).

Metabolite M₁ was an amorphous light yellowish powder. Its HR-ESI-MS exhibited a pseudomolecular ion [M – H]⁻ at m/z 989.3147 (calc. for C₄₃H₅₈O₂₆; 989.3144), compatible with the molecular formula C₄₃H₅₈O₂₆. The IR spectrum showed absorption bands typical of hydroxyls (3414 cm⁻¹), α,β-unsaturated ester (1710; 1630 cm⁻¹), and aromatic rings (1604; 1515 cm⁻¹). Comparison of the ¹H and ¹³C NMR data of M₁ with those of M₆ suggested that they are identical except that M₁ has an additional glucuronide group, which was further supported by M₁ having 176 mass units more than M₆. Meanwhile, the ¹³C NMR data clearly showed the existence of a glucuronic acid [δ 73.1 (C-4‴‴), δ 74.7 (C-2‴‴), δ 76.5 (C-5‴‴), δ 77.3 (C-3‴‴), δ 102.9 (C-1‴‴), and δ 173.3 (C-6‴‴)]. In the HMBC spectrum, the anomeric proton of glucuronic acid (δ 4.93, 1H, d, J = 8.0 Hz) had correlation with C-3‴‴ (δ 147.9), which indicated that the linkage site of glucuronic acid moiety was at C-3‴‴. The β-form anomeric configuration of the glucuronic acid was judged from its coupling constant of the anomeric proton (J = 8.0 Hz). Furthermore, the position of two methoxy groups at C-3 and C-4‴″‴ was confirmed by an NOE experiment in which the OCH₃ resonances at δ 3.79 and δ 3.83 were irradiated. The resulting NOE difference spectrum showed an enhancement of aromatic protons at δ 6.81 (H-2) and δ 6.98 (H-5‴″‴), respectively. On the basis of spectral evidence, mainly 2D-NMR (HSQC, HMBC, and gCOSY), the structure of compound M₁ was elucidated as 2-(3-methoxy-4-hydroxyphenyl)-ethyl O-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-[4-[(2E)-3-(3-O-β-D-glucuronic acid-4-methoxy-phenyl)-2-propenoate]-β-D-glucopyranoside.

Metabolite M₂ was an amorphous light yellowish powder. Its HR-ESI-MS exhibited a pseudomolecular ion [M – H]⁻ at m/z 989.3147 (calc. for C₄₃H₅₈O₂₆; 989.3144), compatible with the molecular formula C₄₃H₅₈O₂₆. The IR spectrum showed absorption bands typical of hydroxyls (3414 cm⁻¹), α,β-unsaturated ester (1710; 1630 cm⁻¹), and aromatic rings (1604; 1515 cm⁻¹). M₂ was 176 mass units higher than that of M₈, suggesting that it was a glucuronide conjugate of M₈. Meanwhile, the ¹³C NMR data clearly showed the existence of a glucuronic acid [δ 73.0 (C-4‴‴), δ 74.6 (C-2‴‴), δ 76.5 (C-5‴‴), δ 77.3 (C-3‴‴), δ 102.9 (C-1‴‴), and δ 173.3 (C-6‴‴)]. In the HMBC spectrum, the anomeric proton of glucuronic acid (δ 4.94, 1H, d, J = 7.5 Hz) had correlation with C-3‴‴ (δ 147.9), which indicated that the linkage site of glucuronic acid moiety was at C-3‴‴. The β-form anomeric configuration of the glucuronic acid was judged from its coupling constant of the anomeric proton (J = 7.5 Hz). Furthermore, the position of two methoxy groups at C-4 and C-4‴″‴ was confirmed by an NOE experiment in which the OCH₃ resonances at δ 3.75 and δ 3.83 were irradiated. The resulting NOE difference spectrum showed an enhancement of aromatic protons at δ 6.77 (H-5) and δ 6.98 (H-5‴″‴), respectively. On the basis of spectral evidence, mainly 2D-NMR (HSQC, HMBC, and gCOSY), the structure of compound M₂ was elucidated as 2-(3-hydroxy-4-methoxyphenyl)-ethyl O-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-[4-[(2E)-3-(3-O-β-D-glucuronic acid-4-methoxy-phenyl)-2-propenoate]-β-D-glucopyranoside.
Fig. 2. Proposed metabolic pathways of ECH.
nyl)-ethyl O-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-[4-[(2E)-3-(3-Oβ-D-glucuronic acid-4-methoxy-phenyl)-2-propenoate]]-β-D-glucopyranoside.

Metabolite M3 was an amorphous light yellowish powder. Its HRESI-MS showed the pseudomolecular ion [M − H]− at m/z 893.2337 (calc. for C37H50O23S; 893.2391). The molecular formula was determined to be C37H50O23S in combination with the 1H and 13C NMR spectral data. The presence of a sulfate moiety was deduced by the appearance of intense distinctive absorption bands at 1268, 1046 cm−1 in the IR spectrum of M4, which was ascribed to the conjugated sulfur- oxygen bond stretching vibration. The IR spectrum also showed absorption bands typical of hydroxyls (3428 cm−1), α,β-unsaturated ester (1702, 1630 cm−1), and aromatic rings (1611; 1514 cm−1). The molecular formula of M4 was 80 mass units higher than that of M8, suggesting that M4 was a sulfate conjugate of M8. The 13C NMR data of M4 were similar to that of those of M8, except for the downfield shifts of C-2 (4.1 ppm), and the upfield shifts of C-3 (−4.3 ppm) and C-5 (+7.5 ppm), and the upfield shifts of C-4 (−4.6 ppm), which showed that the sulfonate group was linked at C-4. On the basis of spectral evidence, mainly 2D-NMR (HSQC and HMBC), the structure of compound M4 was elucidated as 2-(3-sulfo-4-methoxyphenyl)-ethyl O-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-(4-O-E-isof eruloyl)-β-D-glucopyranoside.

Metabolite M4 was an amorphous light yellowish powder. Its HRESI-MS exhibited a pseudomolecular ion [M − H]− at m/z 893.2348 (calc. for C37H50O23S; 893.2391). The molecular formula was determined to be C37H50O23S in combination with the 1H and 13C NMR spectral data. The presence of a sulfate moiety was deduced by the appearance of intense distinctive absorption bands at 1267, 1046 cm−1 in the IR spectrum of M4, which was ascribed to the conjugated sulfur- oxygen bond stretching vibration. The IR spectrum also showed absorption bands typical of hydroxyls (3428 cm−1), α,β-unsaturated ester (1702, 1630 cm−1), and aromatic rings (1611; 1514 cm−1). The molecular formula of M4 was 80 mass units higher than that of M8, suggesting that M4 was a sulfate conjugate of M8. The 13C NMR data of M4 were similar to that of those of M8, except for the downfield shifts of C-2 (+7.4 ppm) and C-4 (+4.1 ppm), and the upfield shifts of C-3 (−4.7 ppm), which showed that the sulfonate group was linked at C-3. On the basis of spectral evidence, mainly 2D-NMR (HSQC and HMBC), the structure of compound M4 was elucidated as 2-(3-sulfo-4-methoxyphenyl)-ethyl O-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-(4-O-E-isof eruloyl)-β-D-glucopyranoside.

**Discussion**

During the drug metabolism studies, it becomes vitally important to identify and characterize the structure of metabolites. In recent years, new techniques were applied to identify and characterize the metabolites, such as gas chromatography-MS, LC-MS (Rüfer et al., 2006; Xu et al., 2006), LC/NMR (Mutlib and Shockcor, 2003), NMR (Zhang et al., 2004; Zeng et al., 2007), and stable isotopes. In our study, three groups of positional isomers (M5, M6, M7, and M8; M1 and M2; and M3 and M4) were obtained. It was very difficult to
identify the structure of these metabolites only by LC/multiple tandem mass spectrometry data. So, preparation of metabolites and further identification based on NMR data must be done. Of course, the direct isolation of the metabolites from bile, urine, or feces of humans or other animals was a tedious and laborious task, but it is the most reliable method in the identification of metabolites.

We have reported the low bioavailability (0.83%) of ECH in rat after oral administration (Jia et al., 2006). The extremely low bioavailability was mainly due to poor absorption of ECH through the gastrointestinal tract. In the present study, we have first investigated the metabolites of ECH from rat bile. Eight metabolites of ECH were isolated and identified was mainly due to poor absorption of ECH through the gastrointestinal tract. In the present study, we have first investigated the metabolites of ECH in rats were reported previously that ECH was hydrolyzed to acteoside by enzymes of bacterial origin in the large intestine of beagle dogs (Lei et al., 2001). In the present study, three types of metabolic pathways for ECH in rats were revealed (Fig. 2). One pathway was the methylation of the phenolic hydroxyl groups in the ester and aglycone for ECH, and four positional isomeric O-dimethyl metabolites were formed. It is interesting to note that the monomethyl metabolites were not isolated and identified, suggesting that phenolic hydroxyls on ester or aglycon are easy to methylate and that the catechol is hard to methylate simultaneously. The second pathway was that the gluracuronidation tends to occur on hydroxyl groups of the ester moiety in ECH. Two isomeric O-dimethyl glucuronides were formed. The third pathway was that the sulfation tends to occur on hydroxyl groups of the aglycone moiety in ECH. Two isomeric O-dimethyl sulfates were identified. In addition, the author also studied the metabolism of PEGs can be found. Our group reported previously that ECH was hydrolyzed to acteoside by enzymes of bacterial origin in the large intestine of beagle dogs (Lei et al., 2001). In the present study, three types of metabolic pathways for ECH in rats were revealed (Fig. 2). One pathway was the methylation of the phenolic hydroxyl groups in the ester and aglycone for ECH, and four positional isomeric O-dimethyl metabolites were formed. It is interesting to note that the monomethyl metabolites were not isolated and identified, suggesting that phenolic hydroxyls on ester or aglycon are easy to methylate and that the catechol is hard to methylate simultaneously. The second pathway was that the glucuronidation tends to occur on hydroxyl groups of the ester moiety in ECH. Two isomeric O-dimethyl glucuronides were formed. The third pathway was that the sulfation tends to occur on hydroxyl groups of the aglycone moiety in ECH. Two isomeric O-dimethyl sulfates were identified. In addition, the author also studied the metabolism of ECH in the gut and the serum in rat. Two metabolites were detected in the gut. And they were identified as acteoside and caffeic acid
Based on their retention times compared with authentic standards and on LC-MS (ESI-tandem mass spectrometry) analyses.

It was reported previously that the preferential conjugation sites existed in the conjugate formation for flavonoid compounds (Morand et al., 1998; Boersma et al., 2002; Havsteen, 2002; Chen et al., 2006; Bursztyka et al., 2003), and caffeic acid and its oligomer condensate (Morandi et al., 2002; Zhang et al., 2004). In the present study, the preferential conjugation sites also existed in the conjugate formation for ECH, which may be species difference. Of the major metabolic pathways to biotransform catechols into conjugates glucuronidation by the enzyme UDP-glucuronosyltransferase, and sulfotransferase (species difference). Of the three types of metabolites in rat, the major conjugates were the methyl ethers and the glucuronides, whereas the sulfates were minor. Among four methyl ethers, the content of M6 was larger than others by comparing their peak area in HPLC chromatogram, which revealed the possibilities of the methylation on 3′′′′-OH and 3-OH in ECH at the same time was larger than that of other hydroxyls. Furthermore, the glucuronidation with a high selectivity only occurred on the 3′′′′-OH group of the ester. So, the 3′′′′-OH group of the ester of ECH was determined as the preferential site for glucuronosyl conjugation compared with other three hydroxyl groups. Moreover, sulfate ester formation occurred at hydroxyl-3 or -4 of the aglycone, whereas the ester contained 3′′′′-OH and 4′′′′-OCH₃. However, the conjugates with mixed methylates/sulfates/glucuronides or sulfate/glucuronides were not found. Further studies should clarify whether they were formed or not.

Metabolism of ECH involves methylation by the enzyme COMT, glucuronidation by the enzyme UDP-glucuronosyltransferase, and sulfation by sulfotransferase in the liver. COMT is an intracellular enzyme, widely distributed throughout the organs of the body, with the highest COMT activity is in the liver, followed by the kidneys and gastrointestinal tract (Karhunen et al., 1994). Some phenolic compounds, such as caffeic acid (Moridi et al., 2002), quercetin (O’Leary et al., 2003), and magnesium lithospermate B (Zhang et al., 2004), were reported to be methylated in the catechol moiety by COMT. In the present study, ECH were methylated in the catechol moiety at the 3′′′′,4′′′′-OH of the ester and at the 3′,4-OH of the aglycone, to form four O-dimethylated conjugates (M5, M6, M7, and M8), respectively, by COMT. In addition, glucuronidation and sulfation reactions may well be a major cause for the lack of toxicity of phenolic compounds (Morand et al., 1998) and seemed to be the other major metabolic pathways to biotransform catechols into conjugates

### Table 3

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**Note:** All spectra were recorded on a Varian INOVA-500 spectrometer, in CD₃OD.

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[^1]: NMR data for metabolites M1-M4 (d in ppm, J in Hz).

[^m]: multiple splenic br, s; broad singlet.
unable to enter a redox cycling reaction for phenolic compounds (Bursztyka et al., 2008). Two O-dimethyl glucuronides and two O-dimethyl sulfates were also identified in the present study. Moreover, the glucuronidation and sulfadation improve the water solubility of methoxylated derivatives of ECH and consequently favor their elimination. Metabolism needs to be further investigated, and the enzymes involved in the formation of metabolites of ECH need to be identified.

In summary, eight phase II metabolites have been isolated and identified from bile collected from rats after oral or intravenous administration of ECH, and four of them were O-dimethyl conjugates, two were O-dimethyl glucuronides, and two were O-dimethyl sulfates. The methylation and conjugation glucuronidation conjugates were major metabolites, whereas sulfation conjugates were minor. The hydroxyl groups of the aglycone and the ester could also be methylated. The preferential site for the glucuronidation was 3""OH of the ester, and the preferential sites for sulfation were 3- or 4-OH of the aglycone. All above-mentioned results are important for the understanding of ECH metabolism in rats and provide information and reference for the further metabolic investigation of ECH in humans.

References


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