From Single Compounds to Herbal Extract: A Strategy to Systematically Characterize the Metabolites of Licorice in Rats

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ABSTRACT:
Because of the complicated chemical composition of traditional Chinese medicines (TCMs), their metabolic study has been a great challenge, especially when they are used in the traditional way, i.e., clinical oral dose of water decoction. Poor understanding of metabolic pathways and too low metabolite concentrations to be detected in biosamples are the major hurdles. In the present work, a three-step strategy was proposed to systematically characterize in vivo metabolites of TCMs at a normal clinical dosage. Licorice, one of the most popular TCMs, was studied as a model. First, 10 representative compounds of licorice were administered to rats separately. A total of 68 metabolites were characterized by high-performance liquid chromatography coupled with diode-array detection and electrospray ionization tandem mass spectrometry and liquid chromatography (LC)/quadrupole time-of-flight-mass spectrometry (MS) analyses, together with enzyme hydrolysis. Among these, 13 compounds were confirmed by comparison with reference standards, including the 10 administered licorice compounds. Second, a high dose (equivalent to 20-fold clinical dosage) of licorice water extract was administered, and 22 more metabolites were characterized. Finally, these metabolites (including constituents of licorice) were determined by a highly sensitive and selective LC/selected reaction monitoring-MS method when the licorice water decoction was orally administered to rats at a clinical dosage (0.9 g crude drug/kg). A total of 42 metabolites in plasma and 62 metabolites in urine were detected. This is the first attempt to fully profile the in vivo metabolites of licorice at a normal clinical dosage.

Introduction
Traditional Chinese medicines (TCMs) have a long history of use in clinical practice, and their therapeutic effects are generally considered credible. However, to determine the active ingredients that play a therapeutic role is not easy because of their complicated chemical composition. One feasible way is to identify the in vivo metabolites because only the exposed chemicals could play this role in most cases. Moreover, to clarify “which are absorbed” (chemical constituents of the TCM per se) and “which are produced” (biotransformed metabolites) is the basis for pharmacokinetic studies. The metabolism of TCMs is a complicated “system to system” process unfortunately and has presented a great challenge (Lan and Jia, 2010). TCMs contain multiple compounds that vary significantly in structural types, physiochemical properties, and relative amounts. When they are used in the traditional manner, i.e., water decoction administered by oral route, a number of compounds may enter the circulation and then be converted into even more metabolites. Identification of these absorbed constituents and their metabolites is usually hindered by their fairly low concentrations in vivo and poor understanding of the metabolic pathway.

Given the above-mentioned difficulties in TCM metabolic studies, most current reports focused on only one or a few single compounds, which could not represent the whole herbal medicine (Qiu, 2010). Other reports used a very high dose of concentrated herbal extracts (40–200 times higher than clinical dosage) to ensure that the metabolites could be detected in biosamples (Wang et al., 2007; Tan et al., 2010). Few efforts to study the metabolism of TCMs under circumstances similar to those in clinical therapy have been made. Furthermore, only a few metabolites were characterized in most reports even when highly sensitive technologies such as LC/MS were used. To systematically characterize the metabolites of TCMs at an oral clinical dosage, a new strategy is needed.

In the present work, we propose a strategy to systematically characterize the in vivo metabolites of TCMs at an oral clinical dosage. An illustrative diagram is shown in Scheme 1. The strategy consisted of...
three steps: 1) characterize the metabolites of representative single compounds of a TCM by LC/MS and then analyze the metabolic pathways of each type of compound; 2) administer a high dosage of herbal extracts and discover more metabolites according to metabolic pathways of the same type of compound; and 3) establish a highly sensitive and selective LC/SRM-MS method and detect all the metabolites in biosamples at a normal clinical dosage. This strategy was applied by using licorice as a model herb.

Licorice was selected as the model herb because it is the most frequently used TCM. It appears in approximately 60% of all TCM prescriptions (Wang and Yang, 2007). Licorice is derived from the roots and rhizomes of *Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, and *Glycyrrhiza glabra* (Chinese Pharmacopoeia Commission, 2010) and is mainly used to treat peptic ulcer, cough, and hepatitis C (Asl and Hosseinzadeh, 2008). To date, more than 400 compounds have been isolated from licorice, and the major constituents are flavonoids and saponins (Zhang and Ye, 2009). The metabolism of licorice has been extensively studied. Most of these studies were focused on single licorice compounds such as glycyrrhizin acid (Makino et al., 2008), liquiritigenin (Shimamura et al., 1993), and isoliquiritigenin (Guo et al., 2008a,b; Cuendet et al., 2010). Some other studies attempted to detect the in vivo metabolites after licorice extracts were administered (Homma et al., 1997; Zuo et al., 2003; Hou et al., 2005). However, only a few compounds were detected in most reports. In a recent study, 25 licorice compounds were detected in rat plasma after a high dose of ethanol extract (40 g/kg) was administered, whereas no biotransformed metabolites were characterized (Tan et al., 2010). To the best of our knowledge, no report is available to provide a full metabolic profile of licorice at a normal dosage.

In this article, the in vivo metabolites of licorice water extract (LWE) in rats at a normal clinical dosage were systematically characterized by our new strategy. First, the metabolism of 10 representative single compounds was studied, and a total of 68 metabolites were characterized. Second, a high dosage (20-fold clinical dosage) of LWE was administered, and 22 more metabolites were characterized. Finally, a normal clinical dosage of LWE was administered to rats, and 42 metabolites were detected in plasma and 62 in urine by a highly sensitive LC/SRM-MS method.

**Materials and Methods**

**Chemicals and Reagents.** Licorice (the dried roots and rhizomes of *G. uralensis* Fisch.) was purchased from Elion Resources Group Company (Erdos, Inner Mongolia, China) and was authenticated by comparison of its HPLC fingerprint with that of a reference sample from the China National Institutes for Food and Drug Control (Beijing, China). Liquiritigenin (LG), liquiritin

![Fig. 1. HPLC fingerprint of licorice water extract (240 nm), showing 10 representative compounds studied in this article. mAU, milli-absorbance units.]
(LQ), liquiritin apioside (LA), isoliquiritigenin (ILG), isoliquiritin (ILQ), isoliquiritin apioside (ILA), ononin (ONO), glycyrrhizic acid (GLY), 7,4'-dihydroxyflavone (DHF), isongustone A (IAA), formononetin (FOR), glycyrrhetinic acid, genkwanin, licoisoflavone A, licoisoflavone B, semilicoisoflavone B, and glycycomarin were isolated from licorice by the authors. The structures were fully characterized by NMR spectroscopy and mass spectrometry. The purities were greater than 98%, determined by HPLC/UV analysis. Daidzein, naringenin, and naringenin dihydrochalcone were purchased from Zelang Co. Ltd. (Nanjing, China). S-Equol, together with β-glucuronidase (HP-1 type, prepared from Helix pomatia having β-glucuronidase activity of 1,926,000 U/g and sulfatase activity of more than 10,000 U/g) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile was from...
<table>
<thead>
<tr>
<th>No.</th>
<th>RT (min)</th>
<th>λ_{max} (nm)</th>
<th>Formula</th>
<th>[M - H]^- (m/z)</th>
<th>Measured</th>
<th>Predicted</th>
<th>Difference</th>
<th>MS/MS^\dagger</th>
<th>Metabolite</th>
<th>Plasma</th>
<th>Urine</th>
<th>Feces</th>
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<td>593.1512</td>
<td>0.3</td>
<td>417, 255, 175</td>
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<td>LQ; LA; D^±</td>
<td>LQ; ILQ; D^±</td>
<td>N.D.</td>
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<td>333</td>
<td>SZ-di-O-Sul</td>
<td>LA; LG; D^±</td>
<td>LQ; LG; D^±</td>
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<td>LQ; LG; LA; ILQ; D^±</td>
<td>N.D.</td>
<td></td>
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</tbody>
</table>

### Table 1: Characterization of in vivo metabolites of 10 single licorice compounds in rats by HPLC-DAD-ESI-MSn and LC/qTOF-MS

- **No.** refers to the compound number.
- **RT** is the retention time in minutes.
- **λ_{max}** is the wavelength of maximum absorbance in nanometers.
- **Formula** is the chemical formula of the metabolite.
- **[M - H]^- (m/z)** are the measured and predicted masses of the metabolite.
- **Measured** and **Predicted** are the measured and predicted values, respectively.
- **Difference** is the absolute difference between measured and predicted values.
- **MS/MS^\dagger** indicates the fragment ions observed in the MS/MS analysis.
- **Metabolite** is the name of the metabolite.
- **Plasma, Urine, Feces** indicate the biological samples where the metabolite was detected.

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**Notes:**
- D^±: Detected in both directions.
- N.D.: Not detected.
- ONO: O-n-Ouabain
- DA: Daunorubicin
- ILQ: Isouvan
- LQ: Louvan
- LG: Lagozine
- ILG: Iliouvan
- LA: Laegrocin
- ILA: Iliouvan-A
- DL: Dihydro-Legozin
- DHF: Dihydrofuroin
- D^-: Detected in negative direction.
- D^+: Detected in positive direction.
- **Equol-O-Sul**: O-Equol sulfated metabolite.

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**References:**
- The table is extracted from a research article on the characterization of licorice metabolites in rats using high-performance liquid chromatography with diode-array detection-electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS) and liquid chromatography with quadrupole time-of-flight mass spectrometry (LC/qTOF-MS).
Mallinkrodt Baker (Phillipsburg, NJ). Ultra-pure water was prepared with a Milli-Q water purification system (Millipore Corporation, Billerica, MA). All other reagents were of analytical grade.

Synthesis of Davidigenin. Davidigenin (DA) was synthesized by hydrogenation of ILG according to the literature (Homma et al., 1997). Fifty milligrams of ILG was dissolved in 10 ml of ethanol and hydrogenated over palladium-charcoal (0.1 g) under nitrogen stirring at room temperature for 2 h. After filtration and evaporation of the solvent, the residue was purified on a silica gel column and eluted with petroleum ether-acetone to obtain DA (43 mg, 86% yield). The MS and $^1$H NMR spectra were consistent with the literature data.

Preparation of LWE. Liquorice drug materials (20 g) were ground into a crude powder, and boiled in 100 ml of water for 1 h twice. The decoctions were combined and concentrated to 10 ml in a vacuum at 50°C. The resulting solution was used as LWE (equivalent to 2 g of crude drug/ml) and was stored at −20°C. For LC/MS analysis, an aliquot of 0.1 ml of LWE was diluted into 1 ml with water and then filtered through a 0.45-μm membrane.

Animals. Male Sprague-Dawley rats (180–220 g) were obtained from the Laboratory Animal Center of Peking University Health Science Center. The rats were housed in a cage (465 × 300 × 200 mm) in a breeding room at 25°C, 60 ± 5% humidity, and a 12-h light-dark cycle for 3 days and were given access to water and normal chow ad libitum. All animals were fasted overnight before experiments. The animal facilities and protocols were approved by the Animal Care and Use Committee of Peking University Health Science Center. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Drug Administration to Rats. The pure compounds, including LG, LQ, LA, ILG, ILQ, ILA, ONO, DHF, and IAA were suspended in 0.5% carboxymethyl cellulose-sodium and were given to rats (n = 2) orally at 40 mg/kg separately. As an exception, GLY was dissolved in water and orally administered to rats (n = 2) at 270 mg/kg. LWE was dosed to rats (n = 2) at 20 g/kg (high dosage) and 0.9 g/kg (normal dosage, equivalent to 9 g of crude drug/day for a 60-kg human), respectively.

Preparation of Plasma Samples. Blood was collected from the angular vein at 0.25, 0.5, 1, 2, 4, and 6 h, and was centrifuged at 4000 rpm for 15 min, respectively. The plasma samples were mixed, and an aliquot of 3 ml was treated with 4 volumes of methanol-acetonitrile (2:1, v/v) to precipitate protein. After centrifuging at 9500 rpm for 5 min, the supernatant was dried in vacuum at 37°C, dissolved in 300 μl of methanol, and then filtered through a 0.45-μm membrane.

Preparation of Urine and Feces Samples. Rats were held in metabolism cages (DXL-D; Keke Medical Model Co. Ltd., Shanghai, China), and urine and feces samples were collected for 24 h. An aliquot of 2 ml of urine was loaded on a pretreated SPE column (Oasis HLB 6 ml; Waters, Milford, MA), washed with 3 ml of water, and then successively eluted with 3 ml of 5% methanol and 5 ml of methanol. The methanol eluate was collected and dried in vacuum at 37°C. The residue was dissolved in 300 μl of methanol and filtered through a 0.45-μm membrane for LC/MS analysis. Feces were dried in air and then ground into a crude powder. The powder (1.0 g) was extracted by 20-ml of methanol in an ultrasonic bath for 30 min. The resulting solution was dried, and the residue was dissolved in 500 μl of methanol and filtered through a 0.45-μm membrane for analysis.

Enzyme Hydrolysis. An aliquot of treated plasma or urine sample solution (100 μl) was dried under nitrogen gas and was mixed with 200 μl of β-glucuronidase solution (containing 14.5 U, in sodium acetate buffer, pH 5.5). The mixture was incubated in a 37°C water bath for 5 h and then treated with 4 volumes of methanol-acetonitrile (2:1) for protein precipitation and centrifuged at 13,500 rpm for 5 min. The supernatant was dried under a gentle nitrogen flow and then dissolved in 100 μl of methanol. The solution was filtered through a 0.45-μm membrane for chemical analysis.

HPLC/DAD/ESI-MS$^+$ Analysis. HPLC/DAD/ESI-MS$^+$ analysis was performed on an Agilent series 1100 HPLC instrument coupled with a Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Samples were separated on an Agilent ZORBAX Extend-C18 column (4.6 × 250 mm, 5 μm) protected with a ZORBAX Extend-C18 guard column (4.6 × 12.5 mm, 5 μm). Column temperature was 30°C. The mobile phase consisted of acetonitrile (A) and water containing 0.03% (v/v) formic acid (B), formed on an Agilent series 1100 HPLC instrument coupled with a Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Samples were separated on an Agilent ZORBAX Extend-C18 column (4.6 × 250 mm, 5 μm) protected with a ZORBAX Extend-C18 guard column (4.6 × 12.5 mm, 5 μm). Column temperature was 30°C. The mobile phase consisted of acetonitrile (A) and water containing 0.03% (v/v) formic acid (B), formed on an Agilent series 1100 HPLC instrument coupled with a Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Samples were separated on an Agilent ZORBAX Extend-C18 column (4.6 × 250 mm, 5 μm) protected with a ZORBAX Extend-C18 guard column (4.6 × 12.5 mm, 5 μm). Column temperature was 30°C.
acid (B). A linear gradient elution program was used as follows: 0 min, 12% A; 30 min, 39% A; 40 min, 44% A; 50 min, 95% A; and 55 min, 95% A. The flow rate was 1.0 ml/min, and the effluent was introduced into the ESI source of the mass spectrometer at 0.25 ml/min via a T-union splitter. DAD spectra were obtained by scanning from 200 to 400 nm. For ESI-MS analysis, ultra-high-purity helium was used as the collision gas and high-purity nitrogen as the nebulizing gas. The optimized parameters in the negative ion mode were as follows: ion spray voltage, 4.5 kV; sheath gas (nitrogen), 50 arbitrary units; auxiliary gas (nitrogen), 10 arbitrary units; capillary temperature, 320°C; capillary voltage, −12 V; and tube lens offset voltage, −40 V. Mass spectra were recorded in the range of m/z 150 to 1000. MS^n (n = 2–4) was triggered by a data-dependent threshold. The collision-induced dissociation energy was adjusted to 32%, and the isolation width of precursor ions was 2.0 mass units.

**LC/qTOF-MS Analysis.** High-accuracy mass spectra were obtained on a 6510 qTOF mass spectrometer coupled to an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA). The HPLC conditions were the same as those for HPLC/DAD/ESI-MS^n analysis. The ESI source was operated in the negative ion mode. High-purity nitrogen was used as both drying gas (10 l/min) and nebulizing gas (40 psig). Gas temperature was 350°C. Other parameters were as follows: capillary voltage, 4000 V; fragmentor voltage, 130 V; skimmer voltage, 65 V; octopole 1 rf voltage, 750 V; and data acquisition, 2 spectra/s. Data were analyzed with qualitative analysis software (MassHunter Workstation; Agilent Technologies).

**LC/SMRM-MS Analysis.** An LC/SMRM-MS method was established to analyze the plasma and urine samples at a normal dosage of LWE. The system consisted of a TSQ Quantum triple quadrupole mass spectrometer interfaced by an ESI probe with a Surveyor LC system controlled by Xcalibur (1.4 SR1) software (Thermo Fisher Scientific). The HPLC conditions were the same as those for HPLC/DAD/ESI-MS^n analysis. The ESI source was operated in the negative ion mode. High-purity nitrogen was used as both drying gas (40 arbitrary units) and auxiliary gas (5 arbitrary units). High-purity argon was used as the collision gas (1.0 mTorr). Ion source parameters were as follows: spray voltage, 4.0 kV; capillary temperature, 330°C; capillary offset, −35 V; and source-fragementation voltage, 10 V. The mass spectrometer was operated in the SRM mode. The ionization parameters were optimized by infusing methanol solutions of representative compounds (LQ, LG, DHF, IAA, and GLY) at a flow rate of 5 µl/min. The SRM ion pairs were set according to MS^n data obtained in HPLC/DAD/ESI-MS^n analysis.

**Licorice Compounds Database.** To help deduce the origin of metabolites, a homemade information database of all compounds isolated from Glycyrrhiza species was established [licorice compounds database (LCD)]. The information was obtained from SciFinder Scholar. A total of 422 compounds were listed in a Microsoft Excel file. Each compound contained the following information: generic name, structure (basic skeleton and substitution groups), molecular weight, UV absorption maximum, plant source, and reference literature (Supplemental Fig. 1).

**Results**

**Characterization of In Vivo Metabolites of 10 Single Licorice Compounds in Rats.** Ten representative compounds of licorice were selected to identify their metabolites. These compounds included three flavanones (LG, LQ, and LA), three chalcones (ILG, ILQ, and ILA), two isoFlavones (ONO and IAA), one flavone (DHF), and one saponin (GLY). They represent different structural types and are also the major constituents of licorice (Figs. 1 and 2). Their UV spectra, together with MS and MS/MS spectra, are given in Supplemental Fig. 2. After oral administration, their metabolites in rat plasma, urine, and feces were characterized by HPLC/DAD/ESI-MS^n and LC/qTOF-MS (Table 1; Supplemental Table 1). The following information for each metabolite was collected for structural elucidation: HPLC retention time, UV spectra from DAD detector, high-accuracy deprotonated molecule provided by qTOF-MS, and MS^n fragment ions obtained from ion trap mass spectrometry. A total of 68 metabolites were characterized. Among them, 13 were identified by comparison with reference standards, including the 10 administered licorice compounds, together with daidzein, davidigenin, and glycyrrhetinic acid. When pure standards were not available, the structures were partially characterized based on their spectral information. Glucuronide and sulfate conjugates produced [M – H – 176]^− and [M – H – 80]^− fragments in the MS/MS spectra, respectively. For glucuronides, the [glucuronic acid – H]^− ion at m/z 175 could also be observed (Holcapek et al., 2008). Structures of these conjugates were further confirmed by enzyme hydrolysis. After being treated with β-glucuronidase and sulfatase, the conjugates could be converted into their corresponding aglycones, most of which were then identified by comparison with reference standards.

**Metabolites of flavanones.** The metabolism of three licorice flavanones, LG (36), LQ (12), and LA (10), was studied. These compounds per se could be detected in plasma samples. In addition, a number of metabolites were characterized. The major reactions included glucuronidation, sulfation, hydrolysis, isomerization, and hydrogenation.

Glucuronidation was the major metabolic reaction for licorice flavanones. ILG-O-GluA (33) and two isomers of LG-O-GluA (11 and 13) were detected as major metabolites in plasma and urine samples when LG, LQ, or LA was administered. The qTOF mass spectra showed [M – H]^− ions at m/z 431, consistent with the molecular formula of C_{12}H_{15}O_{12}N. In ion trap MS^n spectra, the [M – H]^− ions could further lose a glucuronic acid moiety (176 U) to produce the aglycone ion at m/z 255 (Fig. 3). The m/z 255 ion then fragmented into m/z 135, which was in agreement with the retro-Diels-Alder fragmentation for LG or ILG (Supplemental Fig. 2) (Tan et al., 2010). UV spectra were used to differentiate the isomers. Metabolites 11 and 13 showed an absorption maximum typical for flavanones at 270 to 280 nm and were characterized as LG-O-GluAs. Metabolite 33 showed an absorption maximum typical for chalcones at 372 nm and was characterized as ILG-O-GluA (Fig. 3). The above conjugates were confirmed by enzyme hydrolysis. When the LG plasma sample was treated with β-glucuronidase, peaks for metabolites 11, 13, and 33 disappeared, and peaks corresponding to LG and ILG increased remarkably (Fig. 3). Thus, it could be deduced that these metabolites were glucuronides of LG or ILG.

Sulfate conjugates were also generally detected in plasma and urine samples. Metabolites 3, 7, 19, 21, 23, and 41 produced [M – H – 80]^− ions in their MS/MS spectra. By enzyme hydrolysis, they were characterized as sulfate or glucuronide-sulfate conjugates of LG or ILG. Shimamura et al. (1993) had isolated five LG glucuronide and sulfate conjugates from rat bile, which was consistent with our results.

Flavanone glycosides could be converted into their aglycones by hydrolysis. When LA and LQ were given to rats, high amounts of LG were detected in both urine and feces (Table 1). Isomerization into chalcones was also common for flavanones. ILA (24), ILQ (26), and ILG (46) were detected in rat plasma or urine samples when LA, LQ, and LG were administered, respectively. In addition, flavanones could be further metabolized into dihydrochalcones in urine. Several peaks in the LQ urine sample were converted into davidigenin (4,2′,4′-trihydroxydihydrochalcone) after enzyme hydrolysis. The pure compound of davidigenin was chemically synthesized from isoliquiritigenin by palladium-charcoal hydrogenation and thus allowed the structural characterization. In accordance with our results, Homma et al. (1997) had also detected DA from human urine after administration of licorice. The metabolic pathways of licorice flavanones are illustrated in Scheme 2.

**Metabolites of chalcones.** The metabolism of three licorice chalcones, ILG (46), ILQ (26), and ILA (24), was studied. Of interest, the majority of the chalcone metabolites were the same as the flavanones metabolites that we had described. These metabolites included gluc-
uronide conjugates, sulfate conjugates, and glucuronide-sulfate conjugates. The results indicated that chalcones and flavanones could be converted to each other in rats, which was consistent with a literature report (Cuendet et al., 2010). However, this bioconversion did not always take place. For instance, two ILG-O-GluA (18 and 25) were only detected in ILG or ILQ plasma and urine but not in LG or LQ samples. The metabolic pathways of chalcones are proposed in Scheme 2.

**Metabolites of ONO.** ONO is an isoflavone glycoside in LWE. It could be converted into the aglycone formononetin (47) by eliminating the sugar moiety. Formononetin could further lose the methyl group to produce daidzein (34). Then both formononetin and daidzein underwent hydrogenation to produce dihydroformononetin (49), O-demethylangolensin (45), and equol. These phase I metabolites were then transformed into glucuronide or sulfate conjugates (2, 4, 14, 17, 29, 30, 32, and 42). After enzyme hydrolysis, these conjugates were converted into daidzein, formononetin, and S-equol, which were confirmed by comparing with reference standards. In addition, four monohydroxylated products of formononetin (35, 37, 39, and 43) were detected. Their qTOF mass spectra established the molecular formula of C_{16}H_{12}O_{5}. These products had been reported as metabolites of formononetin (Tolleson et al., 2002; Heinonen et al., 2004). Dihydroformononetin, O-demethylangolensin, and S-equol could be produced by bacterial metabolism, and monohydroxylated products could be catalyzed by liver microsomes. A detailed metabolic pathway of ONO is proposed in Scheme 3.

**Metabolites of IAA.** IAA is an isoflavone bearing two isoprenyl groups (Fig. 2). The parent compound (67) was detected in plasma, urine, and feces samples. In addition, 14 metabolites of IAA were detected, including two monoglucuronides (55 and 65), two diglucuronides (50 and 52), one aldehyde derivative (59), four monohydroxylated derivatives (56, 58, 61, and 66), three dihydroxylated derivatives (51, 53, and 54), and two trihydroxylated derivatives (62 and 64). The newly introduced hydroxyl group of 58 may be located at the isoprene methyl group according to MS/MS data (m/z 437 → 407, loss of CH_{2}O). Similar metabolic reactions had been observed for other flavonoids with isoprenyl groups (Yilmazer et al., 2001; Nikolic et al., 2004). A metabolic pathway of IAA is proposed (Supplemental Fig. 3).
(57 and 60) and glycyrrhetinic acid (68), respectively. In addition, a hydroxylated derivative of glycyrrhetinic acid (63) was observed in plasma. It might be hydroxylated at C-22 or C-24, according to the previous report on glycyrrhetinic acid metabolism (Gao et al., 2007). Glycyrrhetinic acid was the predominant metabolite, indicating that hydrolysis was the major metabolic reaction for GLY. A metabolic pathway of GLY is proposed (Supplemental Fig. 5).

Characterization of More In Vivo Metabolites of LWE at a High Dosage. To characterize more LWE metabolites other than those derived from the above 10 compounds, a high dosage of LWE (20 g/kg) was given to rats. Plasma and urine samples were collected for HPLC/DAD/ESI-MSn and LC/qTOF-MS analysis. HPLC/UV chromatograms and LC/MS total ion currents of the dosed samples were compared with blank samples. Peaks that only appeared in dosed samples could be absorbed licorice constituents or metabolites. A total of 22 new licorice constituents or metabolites were characterized in this step (Table 2; Supplemental Table 2).

Characterization of absorbed licorice constituents. In the LC/MS profile of the LWE-dosed plasma sample, two compounds (88 and 89) were not present in the profile of blank plasma, whereas they were present in LWE extract. These two compounds should be absorbed licorice constituents. They gave high-accuracy $[M - H]^{-}$ ions at $m/z$ 983.4488 and 837.3903, respectively, indicating the molecular formula of C$_{48}$H$_{72}$O$_{21}$ and C$_{42}$H$_{62}$O$_{17}$. The $[M - H]^{-}$ ions could both fragment into $m/z$ 351, indicating that they were licorice saponins. When searching our homemade LCD, we found that licorice saponin A3 and licorice saponin G2 matched the above information very well, thus allowing their characterization.

Characterization of new metabolites. In the LC/MS profile of LWE-dosed samples, peaks that were absent in both blank samples and the LWE extract could be biotransformed metabolites of licorice. A total of 20 new metabolites were detected in this step. Most of them were glucuronide or sulfate conjugates. Their structures were partially characterized by UV and mass spectra. The proposed structures were then confirmed by comparison with reference standards of aglycones after enzyme hydrolysis.

Metabolite 71 showed a $[M - H]^{-}$ ion at $m/z$ 447 and a UV absorption maximum at 284 nm. Its formula was established as C$_{21}$H$_{20}$O$_{11}$ by high-accuracy mass spectrometry ($[M - H]^{-}$ $m/z$ 447.0943). The $[M - H]^{-}$ ion produced two fragments at $m/z$ 271 and 175 in MS/MS, implying that it was a glucuronide conjugate. The $m/z$ 271 ion further yielded a fragment at $m/z$ 151 in MS$^{3}$. When we searched our LCD database, no compound with a molecular weight of 272 had been isolated from G. uralensis. However, in the LWE chromatogram we did find a peak with $\lambda_{max}$ at 290 nm and $[M - H]^{-}$ ion at $m/z$ 433, which could fragment into $m/z$ 271 and 151. These fragments were the same as those for metabolite 71. Searching the database again, we found cheroespion (naringenin-4’-O-glucoside, SCHEME 2. Proposed metabolic pathways of licorice flavanones (LG, LQ, and LA) and chalcones (ILG, ILQ, and ILA) in rats. F, in feces; P, in plasma; U, in urine; Sul, sulfate; Api, apioside; Glc, glucoside.
molecular weight 434), of which the aglycone (naringenin) was in line with the above information very well. Thus, we could preliminarily deduce the aglycone of metabolite 71 as naringenin. The plasma sample was then treated with glucuronidase. The peak for 71 disappeared, and a new peak was identified to be naringenin by comparing with a reference standard (Scheme 4). On the basis of the above evidence, metabolite 71 was characterized as naringenin-O-GluA. Because naringenin was not present in LWE, metabolite 71 should be derived from choerospondin, which was hydrolyzed into naringenin and then conjugated with glucuronic acid. The above information, together with the metabolic pathway of flavanones we had summarized from LG, LA, and LQ assisted us to detect seven new metabolites derived from choerospondin, including one glucuronide (72), one sulfate (76), one glucuronide-sulfate conjugate (70), and four dihydrochalcones (69, 73, 74, and 75).

Metabolite 85 showed UV absorption maximum at 280 nm, suggesting the basic skeleton of isoflavone. It gave an [M−H]− ion at m/z 529, and MS/MS fragments at m/z 353 and 175, indicating that it was a glucuronide. Then we searched our LCD database. Nine compounds had a molecular weight of 354, and only one of them was an isoflavone. Thus, the aglycone was tentatively characterized as licoisoflavone A (5,7,2′,4′-tetrahydroxyl-3′-prenyl isoflavone). The urine sample was then treated with glucuronidase. The peak corresponding to 85 disappeared, and a new peak with [M−H]− m/z 353 appeared. By comparison with a reference standard isolated from licorice, the new peak was confirmed to be licoisoflavone A. Therefore, the structure of metabolite 85 was characterized as licoisoflavone A-O-GluA. Furthermore, we also characterized another monoglucuronide (86) and two diglucuronide conjugates (83 and 84) of licoisoflavone A.

Likewise, eight other metabolites were characterized as two cycloclericoflavanone glucuronides (77 and 78), one genknwanin glucuronide-sulfate conjugate (79), three glycyccoumarin glucuronides (80, 81, and 82), one licoisoflavone B glucuronide (87), and one semilicoisoflavone B glucuronide (90).

Analysis of Normal Dosage Biosamples by LC/SRM-MS. In this step, a highly sensitive and selective LC/SRM-MS method was established. The structures of metabolites characterized in the above two steps, as well as their MS/MS spectral data, were used to set the ion pairs for SRM detection. By using this LC/SRM-MS method, a total of 42 metabolites (including licorice constituents) in plasma and 62 in urine were detected after oral administration of LWE at a normal dosage of 0.9 g/kg (Supplemental Figs. 6 and 7).

Discussion
The metabolic studies of TCMs have been challenging because of their complicated chemical composition and fairly low concentrations in biosamples. In this study, we proposed a three-step strategy to fully profile the in vivo metabolites of TCMs. By using licorice as a model herb, we managed to characterize 90 licorice metabolites in rats, including 12 intact licorice compounds and 78 biotransformed metabolites. By using a highly sensitive LC/SRM-MS method, the majority of these metabolites could be detected in rat plasma or urine after oral administration of normal clinical dosage of licorice water extract.

Considering the complicated chemical composition of licorice, we studied the metabolism of 10 single licorice compounds individually as the first step of our strategy. Because only one single compound was fed to rats, the metabolic profiles were easy to elucidate. The metabolites were characterized by a generally used procedure (Holcapek et al., 2008). These 10 compounds were abundant in licorice
Characterization of licorice metabolites in rats after oral administration of a high dosage (20 g/kg) of licorice water extract by HPLC-DAD-ESI-MS^n and LC/qTOF-MS

Metabolites characterized in Table 1 are not included.

<table>
<thead>
<tr>
<th>Deriving Licorice Compound (mol. wt.)</th>
<th>No.</th>
<th>RT</th>
<th>λ_{max} (nm)</th>
<th>Formula</th>
<th>[M + H]^+</th>
<th>MS/MS^a</th>
<th>Metabolite</th>
<th>Plasma</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cherospondin (434)</td>
<td>69^b</td>
<td>10.34</td>
<td>C_{19}H_{34}O_{18}S</td>
<td>529.0652</td>
<td>529.0657</td>
<td>1.0</td>
<td>353, 449</td>
<td>Naringenin dihydrochalcone-O-GluA-O-Sul</td>
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<tr>
<td></td>
<td>70^b</td>
<td>11.74</td>
<td>C_{22}H_{34}O_{21}S</td>
<td>527.0504</td>
<td>527.0501</td>
<td>−0.6</td>
<td>351, 447</td>
<td>Naringenin-O-GluA-O-Sul</td>
<td>D^4, D^5, D^6</td>
</tr>
<tr>
<td></td>
<td>71^b</td>
<td>16.21</td>
<td>C_{22}H_{34}O_{21}S</td>
<td>447.0943</td>
<td>447.0933</td>
<td>−2.2</td>
<td>271, 175</td>
<td>Naringenin-O-GluA</td>
<td>D^4, D^5, D^6</td>
</tr>
<tr>
<td></td>
<td>72^b</td>
<td>16.85</td>
<td>C_{22}H_{34}O_{21}S</td>
<td>447.0949</td>
<td>447.0933</td>
<td>−3.7</td>
<td>271, 175</td>
<td>Naringenin-O-GluA</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>73^b</td>
<td>17.39</td>
<td>C_{22}H_{34}O_{21}S</td>
<td>449.1097</td>
<td>449.1089</td>
<td>−1.7</td>
<td>273</td>
<td>Naringenin dihydrochalcone-O-GluA</td>
<td>D^4, D^5, D^6</td>
</tr>
<tr>
<td></td>
<td>74^b</td>
<td>18.64</td>
<td>C_{22}H_{34}O_{21}S</td>
<td>449.1091</td>
<td>449.1089</td>
<td>−0.4</td>
<td>273</td>
<td>Naringenin dihydrochalcone-O-GluA</td>
<td>N.D.</td>
</tr>
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<td>75^b</td>
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<td>353.0344</td>
<td>353.0337</td>
<td>−2.0</td>
<td>273, 151</td>
<td>Naringenin dihydrochalcone-O-Sul</td>
<td>N.D.</td>
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<tr>
<td>Cyclolicoisoflavone (340)</td>
<td>76^b</td>
<td>25.83</td>
<td>C_{19}H_{34}O_{18}S</td>
<td>515.1557</td>
<td>515.1559</td>
<td>0.4</td>
<td>339, 175</td>
<td>Naringenin-O-GluA</td>
<td>N.D.</td>
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<td></td>
<td>77^b</td>
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<td>515.1557</td>
<td>515.1559</td>
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<td>339, 175</td>
<td>Naringenin-O-GluA</td>
<td>D^4, D^5, D^6</td>
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<td>Genkwanin (284)</td>
<td>78^b</td>
<td>32.51</td>
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<td>539.0504</td>
<td>539.0501</td>
<td>−2.6</td>
<td>459, 357</td>
<td>Glycycoumarin-O-GluA-O-Sul</td>
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<tr>
<td>Glycycoumarin (368)</td>
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<td>17.44</td>
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<td>719.1853</td>
<td>719.1829</td>
<td>−3.4</td>
<td>367, 352</td>
<td>Glycycoumarin-O-GluA-O-Sul</td>
<td>D^4, D^5, D^6</td>
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<tr>
<td></td>
<td>80^b</td>
<td>24.07</td>
<td>C_{19}H_{34}O_{18}S</td>
<td>705.1691</td>
<td>705.1672</td>
<td>−2.6</td>
<td>352, 393</td>
<td>Licoisoflavone A-O-GluA-DH;DL</td>
<td>D^4, D^5, D^6</td>
</tr>
<tr>
<td>Licoisoflavone A (354)</td>
<td>81^b</td>
<td>24.14</td>
<td>C_{19}H_{34}O_{18}S</td>
<td>543.1517</td>
<td>543.1508</td>
<td>−1.7</td>
<td>367, 497</td>
<td>Glycycoumarin-O-GluA-O-Sul</td>
<td>D^4, D^5, D^6</td>
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<tr>
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<td>82^b</td>
<td>25.09</td>
<td>C_{19}H_{34}O_{18}S</td>
<td>543.1507</td>
<td>543.1508</td>
<td>0.3</td>
<td>367, 497</td>
<td>Glycycoumarin-O-GluA-O-Sul</td>
<td>D^4, D^5, D^6</td>
</tr>
<tr>
<td>Licoisoflavone B (352)</td>
<td>83^b</td>
<td>23.21</td>
<td>C_{19}H_{34}O_{18}S</td>
<td>705.1691</td>
<td>705.1672</td>
<td>−2.6</td>
<td>352, 393</td>
<td>Licoisoflavone B-O-GluA-DH;DL</td>
<td>D^4, D^5, D^6</td>
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<tr>
<td>Licoisoflavone-saponin A3 (984)</td>
<td>84^b</td>
<td>24.20</td>
<td>C_{19}H_{34}O_{18}S</td>
<td>705.1693</td>
<td>705.1672</td>
<td>−2.9</td>
<td>529, 658, 353</td>
<td>Licoisoflavone A-O-GluA-DH;DL</td>
<td>D^4, D^5, D^6</td>
</tr>
<tr>
<td>Licoisoflavone-saponin G2 (838)</td>
<td>85^b</td>
<td>31.20</td>
<td>C_{22}H_{34}O_{21}S</td>
<td>529.1359</td>
<td>529.1351</td>
<td>−1.5</td>
<td>353, 175</td>
<td>Licoisoflavone A-O-GluA-DH;DL</td>
<td>D^4, D^5, D^6</td>
</tr>
<tr>
<td>Semilicoisoflavone B (352)</td>
<td>86^b</td>
<td>31.62</td>
<td>C_{22}H_{34}O_{21}S</td>
<td>529.1362</td>
<td>529.1351</td>
<td>−2.0</td>
<td>353, 175</td>
<td>Licoisoflavone A-O-GluA-DH;DL</td>
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<tr>
<td></td>
<td>87^b</td>
<td>34.96</td>
<td>C_{22}H_{34}O_{21}S</td>
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<td>Licoisoflavone B-O-GluA-DH;DL</td>
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<tr>
<td></td>
<td>88^b</td>
<td>25.42</td>
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<td>983.4488</td>
<td>983.4493</td>
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<td>Licoisoflavone A3</td>
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<td></td>
<td>89^b</td>
<td>30.76</td>
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<td>837.3914</td>
<td>0.6</td>
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<td>Licoisoflavone G2</td>
<td>D^4, D^5, D^6</td>
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<td>25.55</td>
<td>C_{22}H_{34}O_{21}S</td>
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<td>527.1195</td>
<td>−1.3</td>
<td>351, 175</td>
<td>Semilicoisoflavone B-O-GluA</td>
<td>D^4, D^5, D^6</td>
</tr>
</tbody>
</table>

**RT:** retention time; **N.D.:** not detected; **Sul:** sulfite; **w:** signal too weak.

*Bold* indicates the base peak (100% intensity). The base peak for [M + H] is not in bold.

*Confirmed by enzyme hydrolysis.*

*DL, detected in LWE high-dose biosamples by LC/MS.*

*DL, detected in LWE normal dosage biosamples by LC/SRM-MS.*

Water extract and may produce the major in vivo metabolites of licorice. The final results proved that our assumption was correct. Among the 90 metabolites we detected, 68 (or 75%) were derived from these 10 compounds. More importantly, these 10 compounds belong to flavanone, chalcones, flavone, isoflavone, prenylated isoflavone, and saponin, respectively, and represent the major types of licorice compounds. Each type of compound had distinct metabolic pathways. For flavanones and chalcones, glucuronidation and sulfation were the major metabolic reactions, which were in line with literature reports (Shimamura et al., 1993; Guo et al., 2008a; Cuendet et al., 2010). Hydrogenation, hydrolysis, and isomerization were important phase I reactions (Homma et al., 1997; Guo et al., 2008b). Previous studies reported the oxidation of LG and ILG in vitro (Nikolic and van Breemen, 2004; Guo et al., 2008b). However, we did not detect these metabolites in rats. For isoflavone glycoside (ONO), hydrolysis, hydrogenation, and sulfation were the major metabolic reactions. Most of these reactions had been reported previously (Tolleson et al., 2001; Heinonen et al., 2004). For prenylated isoflavone, oxidation and glucuronidation were the major metabolic pathway (Yilmazer et al., 2001; Nikolic et al., 2004). For saponins, hydrolysis and hydroxylation were the most important metabolic pathways, which was consistent with previous reports (Gao et al., 2007; Makino et al., 2008). These metabolic pathways could be extrapolated and were critically important in predicting the metabolites of homologous compounds.

The second step of our strategy was to characterize metabolites after administration of a high dosage of LWE (20 g/kg). This step
aimed to discover metabolites other than those derived from the above single compounds. To detect as many metabolites as possible, we used a high dose of LWE in this step (almost 20-fold higher than normal clinical dosage). Tan et al. (2010) recently reported a similar study, characterizing 25 licorice compounds in rat plasma but no metabolites. Here we characterized 20 biotransformed metabolites, assisted by enzyme hydrolysis and metabolic pathways of homologous compounds. The choerospondin case and licoisoflavone A case mentioned above were two typical ones. It should be mentioned that although we detected a lot more metabolites in this step, their structures were not established owing to limited structural information.

The aim of this study was to profile licorice metabolites at an oral clinical dosage (0.9 g/kg, according to the Chinese Pharmacopoeia Commission, 2010). At this low dose, most metabolites could not be detected by conventional HPLC/DAD/ESI-MSn because of their low concentrations. Therefore, in the third step of our strategy, we used a highly sensitive and selective LC/ESI-MS-MS technique to detect the metabolites because tandem mass spectrometry using SRM has a lower limit of detection than scanning mass spectrometry (Supplemental Fig. 8). However, a prerequisite of this technique is that structures of possible metabolites should be known. Fortunately, this knowledge had already been obtained in the first two steps of our strategy. Most of the precharacterized metabolites gave an obvious signal even though the matrix was very complicated and the concentrations were fairly low (Supplemental Figs. 6 and 7).

To mimic TCM clinical use, licorice was cooked in water to obtain the extract. The dosage was 0.9 g/kg in rats, equivalent to 9 g/day for humans. Under these conditions, a total of 42 metabolites (including intact licorice compounds) were detected in rat plasma and 62 in urine by LC/ESI-MS-MS. However, some metabolites discovered in steps 1 and 2, such as licorice-saponin A3, licorice-saponin G2, were not detected in step 3 and most IAA derivatives. Given the good sensitivity of the SRM technique, it was unlikely that these compounds could not be detected. Instead, we consider that single compound dosing or too high a dosage may change the absorption of rat intestine.

By using the above three-step strategy, a total of 90 licorice-derived phytochemicals were detected in vivo. Although the chemical constituents of theophyllene were very complicated, the 90 chemicals were derived from only 19 licorice compounds, which were LA, LQ, LG, ILA, ILQ, ILG, ONO, DIF, AIA, GLY, choerospondin, licoisoflavone A, licoisoflavone B, genkwanin, glycycoumarin, cyclolicoisoflavone, semilicoisoflavone B, licorice saponin A3, and licorice saponin G2. In the high-performance liquid chromatogram of licorice water extract, these compounds were the major peaks (Fig. 1). Thirteen of these 19 compounds had been reported to possess significant pharmacological activities (Supplemental Table 3). For instance, LQ and ILQ showed antidepressive activities in the mouse forced swimming test and tail suspension test, as well as in the chronic variable model herb and applied our strategy by characterizing 90 phytochemicals (including intact licorice compounds) in rats. After an oral administration of licorice water extract at a normal clinical dosage, 42 metabolites were detected in plasma and 62 in urine by a highly sensitive LC/ESI-MS-MS method. As far as we know, this is the first report on full profiling of licorice metabolites in vivo. This strategy could be generally used for the characterization of TCM metabolites.

Acknowledgments

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Authorship Contributions

Participated in research design: Ye and Xiang.
Conducted experiments: Xiang, Qiao, Wang, Li, Miao, and Ye.
Contributed new reagents or analytic tools: Xiang, Qiao, and Ye.
Performed data analysis: Xiang, Qiao, and Ye.
Wrote or contributed to the writing of the manuscript: Ye, Xiang, and Guo.

References


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