DMD #38695

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

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Running title: Characterization of Licorice Metabolites in Rats

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Number of text pages: 32

Number of tables: 2

Number of figures: 7

Number of references: 28

Number of words in the abstract: 222

Number of words in the introduction: 726

Number of words in the discussion: 1096

Abbreviations

DA, davidigenin; DHF, 7,4′-dihydroxyflavone; FOR, formononetin; GluA, glucuronic acid; GLY, glycyrrhizic acid; HPLC/DAD/ESI-MS^n, high-performance liquid chromatography coupled with diode-array detection and electrospray ionization tandem mass spectrometry; IAA, isoangustone A; ILA, isoliquiritin apioside; ILG, isoliquiritigenin; ILQ, isoliquiritin; LA, liquiritin apioside; LC/SRM-MS, liquid chromatography-selected reaction monitoring mass spectrometry; LG, liquiritigenin; LQ, liquiritin; LWE, licorice water extract; ONO, ononin; Sul, sulfate; TCM, Traditional Chinese Medicine; qTOF-MS, Quadrupole time-of-flight mass spectrometry; XIC, extracted ion chromatogram.

Abstract

Due to 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 bio-samples 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. Firstly, ten representative compounds of licorice were administered to rats separately. A total of 68 metabolites were characterized by HPLC/DAD/ESI-MS^n and LC/qTOF-MS analyses, together with enzyme hydrolysis. Among them, 13 compounds were confirmed by comparison with reference standards, including the 10 administered licorice compounds. Secondly, a high dosage (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/SRM-MS method when licorice water decoction was orally administered to rats at a clinical dosage (0.9 g crude drug per kilogram). 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 been used in clinical practice for a long history, and their therapeutic effects are generally considered credible. However, to find out the active ingredients that play a therapeutic role is not easy due to their complicated chemical composition. One feasible way is to identify the *in vivo* metabolites because only the exposed chemicals could play the role, in most cases. Moreover, to clarify "what are absorbed" (chemical constituents of the TCM *per se*) and "what are produced" (biotransformed metabolites) is the basis for pharmacokinetic studies. Unfortunately, the metabolism of TCMs is a complicated "system to system" process, and has been a great challenge (Lan and Jia, 2010). TCMs contain multiple compounds which 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 get into circulation and then be converted into even more metabolites. Identification of these absorbed constituents and their metabolites are usually hindered by their fairly low concentrations *in vivo* and poor understanding in 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). Some other reports used very high dosage of concentrated herbal extracts (40 to 200 times higher than clinical dosage) to make sure the metabolites could be detected in bio-samples (Wang et al., 2007; Tan et al., 2010). Few efforts have been made to study the metabolism of TCMs under circumstances similar to

clinical therapy. Furthermore, only a few metabolites were characterized in most reports even when highly sensitive technologies like LC/MS were used. In order 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, (i) characterize the metabolites of representative single compounds of a TCM by LC/MS, and then analyze the metabolic pathways of each type of compounds; (ii) administer a high dosage of herbal extracts and discover more metabolites according to metabolic pathways of the same type of compounds; (iii) establish a highly sensitive and selective LC/SRM-MS method, and detect all the metabolites in bio-samples at a normal clinical dosage. This strategy was realized 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 about 60% of all TCM prescriptions (Wang and Yang, 2007). Licorice is derived from the roots and rhizomes of *Glycyrrhiza uralensis*, *G. inflata*, and *G. glabra* (Chinese Pharmacopoeia Commission, 2010), and is mainly used to treat peptic ulcer, cough, and hepatitis C (Asl and Hosseinzadeh, 2008). Up 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 like glycyrrhizic acid (Makino et al., 2008),

liquiritigenin (Shimamura et al., 1993), and isoliquiritigenin (Guo et al., 2008a, 2008b; Cuendet et al., 2010). Some other studies attempted to detect the *in vivo* metabolites after licorice extracts were administered (Homme et al., 1997; Zuo et al., 2003; Hou et al., 2005). However, only a few compounds were detected in most reports. Recently, 25 licorice compounds were detected in rat plasma after a high dosage of ethanol extract (40 g/kg) was administered, while 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 paper, the *in vivo* metabolites of licorice water extract (LWE) in rats at a normal clinical dosage were systematically characterized by our new strategy. Firstly, the metabolism of ten representative single compounds was studied, and a total of 68 metabolites were characterized. Secondly, 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 *Glycyrrhiza uralensis* Fisch.) was purchased from Elion Resources Group Company (Inner Mongolia, China), and was authenticated by comparing its HPLC fingerprint with that of a reference sample from China National Institutes for Food and Drug Control (Beijing, China). Liquiritigenin (LG), liquiritin (LQ), liquiritin apioside (LA), isoliquiritigenin (ILG), isoliquiritin (ILQ), isoliquiritin apioside (ILA), ononin (ONO), glycyrrhizic acid (GLY), 7,4′-dihydroxyflavone (DHF), isoangustone A (IAA), formononetin (FOR), glycyrrhetinic acid, genkwanin, licoisoflavone A, licoisoflavone B, semilicoisoflavone B, and glycycoumarin were isolated from licorice by the authors. The structures were fully characterized by NMR spectroscopy and mass spectrometry. The purities were above 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 *Helixa pomatia* having a *β*-glucuronidase activity of 1,926,000 U/g and a sulfatase activity of over 10,000 U/g) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile was from J.T. Baker (Philipsburg, NJ, USA). Ultra-pure water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

Synthesis of davidigenin

Davidigenin (DA) was synthesized by hydrogenation of isoliquiritigenin (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 vigorous 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 licorice water extract (LWE)

Licorice 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

vacuum at 50°C. The resulting solution was used as LWE (equivalent to 2 g 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 dark-light

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 Animal Care and Use Committee of Peking University Health Science Center.

All procedures were in accordance with the National Institute of Health guidelines

regarding the principles of animal care (2004).

Drug administration to rats

The pure compounds, including LG, LQ, LA, ILG, ILQ, ILA, ONO, DHF and IAA were suspended in 0.5% CMC-Na, 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 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 4,000 rpm for 15 min, respectively. The plasma samples were mixed, and an aliquot of 3 mL was treated with four volumes of methanol-acetonitrile (2:1, v/v) to precipitate protein. After centrifuging at 9,500 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), and urine and feces samples were collected for 24 h. An aliquot of 2 mL of urine was loaded on a pre-treated SPE column (Oasis HLB 6 cc, Waters), 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-fold 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, sodium acetate buffer, pH 5.5). The mixture was incubated in a 37°C water bath for 5 h and then treated with four volumes of methanol-acetonitrile (2:1) for protein precipitation. Centrifuge 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^n analysis

HPLC/DAD/ESI-MS^n analysis was performed on an Agilent series 1100 HPLC instrument coupled with a Finnigan LCQ Advantage ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Samples were separated on an Agilent Zorbax Extent-C$_{18}$ column (4.6 × 250 mm, 5 μm) protected with a Zorbax Extend-C$_{18}$ guard column (4.6 × 12.5 mm, 5 μm). Column temperature was 30ºC. The mobile phase consisted of acetonitrile (A) and water containing 0.03% (v/v) formic 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; 55 min, 95% A. The flow rate was 1.0 mL/min and the effluent was introduced into the ESI source of mass spectrometer at 0.25 mL/min via a T-union splitter. DAD spectra were obtained by scanning from 200-400 nm. For ESI-MS analysis, ultra-high purity helium (He) was used as the collision gas and high purity nitrogen (N_2) as the nebulizing gas. The optimized parameters in the negative ion mode were as follows: ion spray voltage, 4.5 kV; sheath gas (N_2), 50 arbitrary units; auxiliary gas (N_2), 10 units; capillary temperature, 320ºC; capillary voltage, -12 V; tube lens offset voltage, -40 V. Mass spectra were recorded in the range of *m/z* 150-1000. Tandem mass spectrometry (MS^n, n=2-4) was triggered by a data-dependent threshold. The collision induced dissociation (CID) 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 (Agilent, USA). 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 (N_2) 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; OCT 1 RF voltage, 750 V; data acquisition, 2 spectra/s. Data were analyzed with a Qualitative Analysis software (MassHunter Workstation, Agilent, USA).

LC/SRM-MS analysis

An LC/SRM-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 (ThermoFisher, San Jose, 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 (N_2) was used as both sheath gas (40 arb) and auxiliary gas (5 arb). High purity argon (Ar) 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; source-fragmentation voltage, 10 V. The mass spectrometer was operated in selected reaction monitoring (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 (LCD)

To help deduce the origin of metabolites, a home-made information database of all compounds isolated from *Glycyrrhiza* species was established (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 literatures (Supplemental Figure 1).

RESULTS

Characterization of *in vivo* metabolites of ten 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 (Fig. 1 and Fig. 2). Their UV spectra, together with MS and MS/MS spectra are given as supplemental data (Supplemental Figure 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 comparing 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 comparing 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 $C_{21}H_{20}O_{10}$. 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 RDA fragmentation for LG or ILG (Supplemental Figure 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 270-280 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 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. had isolated five LG glucuronide and sulfate conjugates from rat bile (Shimamura et al., 1993), 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, DA) after enzyme hydrolysis. The pure compound of davidigenin was chemically synthesized from isoliquiritigenin by Pd-C hydrogenation, and thus allowed the structural characterization. In accordance to our results, Homma et al. had also detected DA from human urine after administration of licorice (Homma et al., 1997). 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. Interestingly, majority of the chalcone metabolites were the same as the flavanones metabolites which we had described. These metabolites included glucuronide 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 literature report (Cuendet et al., 2010). However, this bioconversion did not always take place. For instance, two ILG-*O*-GluAs (**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

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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, fourteen metabolites of IAA were detected, including two monoglucuronides (**55** and **65**), two diglucuronides (**50** and **52**), one aldehyde derivative (**59**), four momohydroxylated 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 well be located at the isoprene methyl group according to MS/MS data (m/z 437→407, loss of CH_2O). Similar metabolic reactions had been observed for other flavonoids with isoprenyl groups (Yilmazer et al., 2001; Nikolic et al., 2004a). A metabolic pathway of IAA is proposed (Supplemental Figure 3).

Metabolites of DHF

DHF (**27**) was poorly absorbed into circulation after oral administration. DHF itself was detected in urine at a low concentration, and two glucuronides (**5** and **8**) were detected in plasma and urine at trace amounts (Supplemental Figure 4). In contrast, DHF was abundant in feces, indicating it had poor bioavailability.

Metabolites of GLY

GLY (**48**) was the most abundant saponin in LWE (Fig. 1). When GLY was orally administered to rats, it was detected in both plasma and feces. GLY could lose one or two molecules of glucuronic acid to produce glycyrrhetinic acid-*O*-GluA (**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-22α 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 Figure 5).

Characterization of more *in vivo* metabolites of LWE at a high dosage

In order to characterize more LWE metabolites other than those derived from the above ten compounds, a high dosage of LWE (20 g/kg) was given to rats. Plasma and urine samples were collected for HPLC/DAD/ESI-MS[n] 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 LWE dosed plasma sample, two compounds (**88** and **89**)

were not present in the profile of blank plasma while 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 home-made licorice compound

database (LCD), we found that licorice-saponin A3 and licorice-saponin G2 matched the

above information very well, and thus allowed their characterization.

Characterization of new metabolites

In the LC/MS profile of LWE dosed samples, peaks that were absent in both blank

samples and 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 comparing with reference standards of

aglycones after enzyme hydrolysis.

Metabolite **71** showed a [M-H]$^-$ ion at *m/z* 447 and 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 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 Da had been isolated from *G. uralensis*. However, in the LWE chromatogram we did

find a peak with λ_{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 choerospondin (naringenin-4'-*O*-glucoside, MW 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). Based on

the above evidence, metabolite **71** was characterized as naringenin-*O*-GluA. Since

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 glucuronides (**72**), one sulfate (**76**), one glucuronide-sulfate

conjugate (**70**), and four dihydrochalcones (**69**, **73**, **74**, **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. Fortunately, 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 comparing 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 cyclolicoflavanone glucuronide (**77** and **78**), one genkwanin glucuronide-sulfate conjugate (**79**), three glycycoumarin glucuronides (**80**, **81** and **82**), one licoisoflavone B glucuronide (**87**), and one semilicoisoflavone B glucuronide (**90**).

Analysis of normal dosage bio-samples 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 selected reaction monitoring (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 Figure 6) (Supplemental Figure 7).

Discussion

The metabolic studies of TCMs have been challenging due to their complicated chemical composition and fairly low concentrations in bio-samples. 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, 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 ten single licorice compounds individually as the first step of our strategy. As only one single compound was fed to rats, the metabolic profiles were easy to be elucidated. The metabolites were characterized by a generally used procedure (Holcapek et al., 2008). These ten compounds were abundant in licorice water extract, and might well 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 ten compounds. More importantly, these ten compounds belong to flavanone, chalcones, flavone, isoflavone, prenylated isoflavone, and saponin, respectively, and represent the major types of licorice compounds. Each type of compounds had distinctive 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., 2008b; Cuendet et al., 2010). Hydrogenation, hydrolysis and isomerization were important phase I reactions (Homma et al., 1997; Guo et al., 2008a). Previous studies reported the oxidation of LG and ILG *in vitro* (Nikolic and Breemen, 2004b; Guo et al., 2008a). 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 previously reported (Tolleson et al. 2002; Heinonen et al., 2004). For prenylated isoflavone, oxidation and glucuronidation were the major metabolic pathway (Yilmazer et al., 2001; Nikolic et al., 2004a). For saponins, hydrolysis and hydroxylation were the most important metabolic pathway, 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. In order to detect as many metabolites as possible, we used a high dosage of LWE in this step (almost 20-fold higher than normal clinical dosage). Recently, Tan et al. reported a similar study, and characterized 25 licorice compounds in rat plasma but no metabolites (Tan et al., 2010). Here we characterized 20 biotransformed metabolites, assisted by enzyme hydrolysis and metabolic pathways of homologous compounds. The cheorospondin 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 due to limited structural information.

This study aimed to profile licorice metabolites at an oral clinical dosage (0.9 g/kg, according to Chinese Pharmacopoeia). At this low dose, most metabolites could not be detected by conventional HPLC/DAD/ESI-MS^n due to their low concentrations. Therefore, in the third step of our strategy, we used a highly sensitive and selective LC/SRM-MS technique to detect the metabolites since tandem mass spectrometry using SRM has a lower limit of detection than scanning mass spectrometry (Supplemental Figure 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 pre-characterized metabolites gave an obvious signal even though the matrix was very complicated and the concentrations were fairly low (Supplemental Figure 6) (Supplemental Figure 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 per day for human. Under these conditions, a total of 42 metabolites (including intact licorice compounds) were detected in rat plasma and 62 in urine by LC/SRM-MS. However, some metabolites discovered in steps one and two were not detected in step three, like licorice-saponin A3, licorice-saponin G2, and most IAA derivatives. Given the good sensitivity of SRM technique, it was unlikely these compounds could not be detected. Instead, we consider that single compound dosing or too

high 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 licorice were very complicated, the 90 chemicals were derived from only 19 licorice compounds, which were LA, LQ, LG, ILA, ILQ, ILG, ONO, DHF, IAA, GLY, cheorospondin, licoisoflavone A, licoisoflavone B, genkwanin, glycycoumarin, cyclolicoflavanone, semilicoisoflavone B, licorice-saponin A3, and licorice-saponin G2. In the HPLC 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 anti-depression activities in mice forced swimming test and tail suspension test, as well as in chronic variable stress induced depression rat model (Wang et al., 2008; Zhao et al., 2008). LA, LQ and LG showed antitussive activities in capsaicin-induced cough model (Kamei et al., 2003). Aside from these extensively studied compounds, less well-known licorice compounds also produced major metabolites. For example, three metabolites were derived from glycycoumarin, which was present in LWE at a noticeable amount. Glycycoumarin had been reported to show potent antispasmodic activities on carbamylcholine-induced mouse jejunum contraction (Sato et al., 2006). A famous TCM formula Shaoyao-Gancao Decoction (composed of licorice and peony root) is widely used to treat spasm and abdominal pain (Katsura, 1995). Our results indicated that glycycoumarin might be an effective component of this formula.

In conclusion, we proposed a new strategy to systematically characterize the *in vivo* metabolites of TCMs. We selected licorice as a model herb, and realized 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/SRM-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

We would like to thank Dr. Tao Bo (Agilent Technologies) for his technical assistance in high accuracy mass spectrometry determination.

Authorship Contributions

Participated in research design: Ye and Xiang.

Conducted experiments: Xiang, Qiao, Wang, Li, Miao, and Ye.

Contributed new reagents or analytical 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

Asl MN and Hosseinzadeh H (2008) Review of pharmacological effects of *Glycyrrhiza* sp. and its bioactive compounds. *Phytother Res* **22**: 709-724.

Chinese Pharmacopoeia Commission (2010) *Pharmacopoeia of the People's Republic of China, vol. 1, 2010 ed.*, p 80, Chinese Medical Science and Technology Press, Beijing.

Cuendet M, Guo J, Luo Y, Chen SN, Oteham CP, Moon RC, Breemen RB, Marler LE, and Pezzuto JM (2010) Cancer chemopreventive activity and metabolism of isoliquiritigenin, a compound found in licorice. *Cancer Prev Res* **3**: 221-232.

Gao K, Wei Y, Yang J, and Duan XH (2007) CYP3A1/2 and 2C9/10 in rat liver microsomes mediate 22α- and 24-hydroxylation of 18α-glycyrrhetinic acid. *Chin Clin Pharmacol Ther* **12**: 1255-1260.

Guo J, Liu DT, Nikolic D, Zhu DW, Pezzuto JM, and Breemen RB (2008a) *In vitro* metabolism of isoliquiritigenin by human liver microsomes. *Drug Metab Dispos* **36**: 461-468.

Guo J, Liu A, Cao HM, Luo Y, Pezzuto JM, and Breemen RB (2008b) Biotransformation of the chemopreventive agent 2',4',4-trihydroxychalcone (isoliquiritigenin) by UDP-glucuronosyltransferases. *Drug Metab Dispos* **36**: 2104-2112.

Heinonen SM, Wahala K, and Adlercreuz H (2004) Identification of urinary metabolites of the red clover isoflavones formononetin and biochanin A in human subjects. *J Agric Food Chem* **52**: 6802-6809.

Holcapek M, Kolarova L, and Nobilis M (2008) High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of

phase I and phase II drug metabolites. *Anal Bioanal Chem* **391**: 59-78.

Homma M, Oka K, Taniguchi C, Niitsuma T, and Hayashi T (1997) Systematic analysis of post-administrative Saiboku-to urine by liquid chromatography to determine pharmacokinetics of Traditional Chinese Medicine. *Biomed Chromatogr* **11**: 125-131.

Hou YC, Hsiu SL, Ching H, Lin YT, Tsai SY, Wen KC, and Chao LPD (2005) Profound difference of metabolic pharmacokinetics between pure glycyrrhizin and glycyrrhizin in licorice decoction. *Life Sci* **76**: 1167-1176.

Kamei J, Nakamura R, Ichiki H, and Kubo M (2003) Antitussive principles of Glycyrrhizae Radix, a main component of the Kampo preparation Bakumondo-to (Mai-men-dong-tang). *Eur J Pharmacol* **469**: 159-163.

Katsura T (1995) The remarkable effect of Kanzo-to and Shakuyakukanzo-to in the treatment of acute abdominal pain. *Japan J Orien Med* **46**: 293-299.

Lan K and Jia W (2010) An integrated metabolomics and pharmacokinetics strategy for multi-component drugs evaluation. *Curr Drug Metab* **11**: 105-114.

Makino T, Ohtake N, Watanabe A, Tsuchiya N, Imamura S, Iizuka S, Inoue M, and Mizukami H (2008) Down-regulation of a hepatic transporter multidrug resistance-associated protein 2 is involved in alteration of pharmacokinetics of glycyrrhizin and its metabolites in a rat model of chronic liver injury. *Drug Metab Dispos* **36**: 1438-1443.

Nikolic D, Li YM, Chadwick LR, Grubjesic S, Schwab P, Metz P, and Breemen RB (2004a) Metabolism of 8-prenylnarigenin, a potent phytoestrogen from hops (*Humulus lupulus*), by human liver microsomes. *Drug Metab Dispos* **32**: 272-279.

Nikolic D and Breemen RB (2004b) New metabolic pathways for flavanones catalyzed by rat liver microsomes. *Drug Metab Dispos* **32**:387-397.

Qiu F (2010) *In vivo* metabolism of chemical constituents of Traditional Chinese Materia Medica: research and discussion. J Int Pharm Res **37**: 321-328.

Sato Y, Akao T, He JX, Nojima H, Kuraishi Y, Morota T, Asano T, and Tani T (2006) Glycycoumarin from Glycyrrhizae Radix acts as a potent antispasmodic through inhibition of phosphodiesterase 3. *J Ethnopharmacol* **105**: 409-414.

Shimamura H, Suzuki H, Hanano M, Suzuki A, and Sugiyama Y (1993) Identification of tissues responsible for the conjugative metabolism of liquiritigenin in rats: an analysis based on metabolite kinetics. *Biol Pharm Bull* **16**: 899-907.

Tan GG, Zhu ZY, Zhang H, Zhao L, Liu Y, Dong X, Lou ZY, Zhang GQ, and Chai YF (2010) Analysis of phenolic and triterpenoid compounds in licorice and rat plasma by high-performance liquid chromatography diode-array detection, time-of-flight mass spectrometry and quadrupole ion trap mass spectrometry. *Rapid Commun Mass Spectrom* **24**: 209-218.

Tolleson WH, Doerge DR, Churchwell MI, Marques MM, and Roberts DW (2002) Metabolism of biochanin A and formononetin by human liver microsomes *in vitro*. *J Agric Food Chem* **50**: 4783-4790.

Wang P, Liang YZ, Zhou N, Chen BM, Yi LZ, Yu Y, and Yi ZB (2007) Screening and analysis of the multiple absorbed bioactive components and metabolites of Dangguibuxue decoction by the metabolic fingerprinting technique and liquid

chromatography/diode-array detection mass spectrometry. *Rapid Commun Mass Spectrom* **21**: 99-106.

Wang WX, Hu XY, Zhao ZY, Liu P, Hu YC, Zhou JP, Zhou DF, Wang ZB, Guo DA, and Guo HZ (2008) Antidepressant-like effects of liquiritin and isoliquiritin from *Glycyrrhiza uralensis* in the forced swimming test and tail suspension test in mice. *Prog Neuro-Psychoph* **32**: 1179-1184.

Wang YC and Yang YS (2007) Simultaneous quantification of flavonoids and triterpenoids in licorice using HPLC. *J Chromatogr B* **850**: 392-399.

Yilmazer M, Stevens JF, and Buhler DR (2001) *In vitro* glucuronidation of xanthohumol, a flavonoid in hop and beer, by rat and human liver microsomes. *FEBS Lett* **491**: 252-256.

Zhang QY and Ye M (2009) Chemical analysis of the Chinese herbal medicine Gan-Cao (licorice). *J Chromatogr A* **1216**: 1954-1969.

Zhao ZY, Wang WX, Guo HZ, and Zhou DF (2008) Antidepressant-like effect of liquiritin from *Glycyrrhiza uralensis* in chronic variable stress induced depression model rats. *Behav Brain Res* **194**: 108-113.

Zuo F, Zhou ZM, Zhang Q, Mao D, Xiong YL, Wang YL, Yan MZ, and Liu ML (2003) Pharmacokinetic study on the multi-constituents of Huangqin-Tang Decoction in Rats. *Biol Pharm Bull* **26**: 911-919.

Footnotes

This work was supported by the 985 Project of Peking University [Grant 985-2-119-121] and the National Science & Technology Mega Project for Primary Drug Innovation from Ministry of Science and Technology of China [Grant 2009ZX09304-002].

Legends for figures

Scheme 1. A three-step strategy to systematically characterize the *in vivo* metabolites of TCM at a normal clinical dosage.

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; LA, liquiritin apioside; LQ, liquiritin; LG, liquiritigenin; ILA, isoliquiritin apioside; ILQ, isoliquiritin; ILG, isoliquiritigenin.

Scheme 3. A proposed metabolic pathway of ononin (ONO) in rats.

Scheme 4. Characterization of choerospondin-derived metabolites in rats after oral administration of high dosage (20 g/kg) of licorice water extract (LWE). XIC, extracted ion chromatogram of LC/MS profile.

FIG. 1. HPLC fingerprint of licorice water extract (240 nm), showing ten representative compounds studied in this paper. DHF, 7,4'-dihydroxyflavone; GLY, glycyrrhizic acid; IAA, isoangustone A; ILA, isoliquiritin apioside; ILG, isoliquiritigenin; ILQ, isoliquiritin; LA, liquiritin apioside; LG, liquiritigenin; LQ, liquiritin; ONO, ononin.

FIG. 2. Chemical structures of licorice compounds.

FIG. 3. Characterization of glucuronide conjugates in rat plasma after oral administration of liquiritigenin (LG). A. Extracted ion chromatograms (XIC) of monoglucuronide conjugates of LG and ILG in plasma before (a) and after (b) β-glucuronidase hydrolysis, and of reference standards (c); B. ESI-MS^n spectra of **13**; C. UV spectra of **11**, **13**, and **33**. LG, liquiritigenin; ILG, isoliquiritigenin, GluA, glucuronic acid.

Table 1. Characterization of *in vivo* metabolites of ten single licorice compounds in rats by HPLC-DAD-ESI-MS^n and LC/qTOF-MS.

No.	RT (min)	λ_{max} (nm)	Formula	[M-H]$^-$ (m/z) Measured	Predicted	Diff.(ppm)	MS/MS	Metabolite	Plasma	Urine	Feces
1[Δ#]	6.51	270	$C_{27}H_{30}O_{15}$	593.1510	593.1512	0.3	**417**; 255; 175	LQ-7-*O*-GluA	LQ; LA; D^L	LQ; ILQ; D^L	nd
2[Δ]	6.84	/	$C_{15}H_{10}O_{10}S_2$	412.9647	412.9643	-1.1	**333**	DZ-di-*O*-Sul	D^L	ONO; D^L	nd
3[Δ#]	8.79	274	$C_{21}H_{20}O_{13}S$	511.0558	511.0552	-1.2	431; **335**	LG-*O*-GluA-*O*-Sul	LA; LG; D^L	LQ; LG; D^L	nd
4[Δ]	8.95	256	$C_{15}H_{10}O_{10}S_2$	412.9638	412.9643	1.1	**333**	DZ-di-*O*-Sul	nd	ONO; D^L	nd
5[Δ#]	9.33	330	$C_{21}H_{18}O_{10}$	429.0831	429.0827	-1.0	**253**; 175	DHF-*O*-GluA	DHF	DHF; D^L	nd
6[Δ#]	9.52	/	$C_{21}H_{22}O_{12}S$	497.0767	497.0759	-1.5	**417**	LQ-7-*O*-Sul	LA; ILA; D^L	D^L	nd
7[Δ#]	9.58	264	$C_{21}H_{20}O_{13}S$	511.0554	511.0552	-0.5	431; **335**	LG-*O*-GluA-*O*-Sul	LQ	LQ; LA; ILQ; D^L	nd
8[Δ#]	9.98	320	$C_{21}H_{18}O_{10}$	429.0824	429.0827	0.2	**253**; 175	DHF-*O*-GluA	DHF; D^L	DHF; D^L	nd
9[Δ#]	10.51	264	$C_{15}H_{14}O_7S$	337.0389	337.0387	-0.4	**217**; 137	DA-*O*-Sul	nd	LQ; LG; ILQ	nd
10[*#]	12.1	272	$C_{26}H_{30}O_{13}$	549.1611	549.1614	0.5	**255**; 417	LA	LA; ILA; D^L	LA; ILA; D^L	ILA
11[Δ#]	12.48	270	$C_{21}H_{20}O_{10}$	431.0977	431.0984	1.6	**255**; 175	LG-*O*-GluA	LQ; LG; LA;	LQ; LG; LA; ILG;	nd

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									ILQ; D^L	D^L	
$12^{*\#}$	12.49	278	$C_{21}H_{22}O_9$	417.1192	417.1191	-0.2	**255**; 135	LQ	LQ; LA; ILA; D^L	LQ; ILQ; D^L	nd
$13^{\Delta\#}$	13.01	278	$C_{21}H_{20}O_{10}$	431.0976	431.0984	1.8	**255**; 175	LG-*O*-GluA	LQ; LG; LA; ILQ; D^L	LQ; LG; ILQ; LA; ILG; D^L	nd
14^{Δ}	13.60	/	$C_{15}H_{10}O_7S$	333.0076	333.0074	-0.5	**253**	DZ-*O*-Sul	ONO	ONO	nd
$15^{\Delta\#}$	14.09	268	$C_{21}H_{22}O_{10}$	433.1150	433.1140	-2.3	**257**; 175	DA-*O*-GluA	nd	LQ; LG; LA; ILQ; ILG; ILA; D^L	nd
$16^{\Delta\#}$	14.26	268	$C_{21}H_{22}O_{13}S$	513.0706	513.0708	0.5	**337**; 433	DA-*O*-GluA-*O*-Sul	D^L	LQ; LG; LA; ILG; ILA; D^L	nd
$17^{\Delta\#}$	14.36	248	$C_{15}H_{10}O_7S$	333.0080	333.0074	-1.7	**253**	DZ-*O*-Sul	ONO; D^L	ONO; D^L	nd
$18^{\Delta\#}$	14.60	346	$C_{21}H_{20}O_{10}$	431.0980	431.0984	0.9	**255**; 175	ILG-O-GluA	ILQ; ILG	ILQ; ILG	nd
$19^{\Delta\#}$	15.16	364	$C_{21}H_{20}O_{13}S$	511.0558	511.0552	-1.2	**431**; 255	ILG-*O*-GluA-*O*-Sul	LQ; ILG; LA	ILG	nd
$20^{\Delta\#}$	15.73	274	$C_{21}H_{22}O_{13}S$	513.0692	513.0708	3.3	**433**; 337; 257	DA-*O*-GluA-*O*-Sul	LA; D^L	LQ; LA; ILQ;	nd

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										ILG; D^L	
$21^{\Delta\#}$	16.34	274	$C_{15}H_{12}O_7S$	335.0239	335.0231	-2.3	**255**	LG-O-Sul	LA; ILG; D^L	LQ; ILG; LG; LA; ILQ; D^L	nd
$22^{\Delta\#}$	16.86	264	$C_{15}H_{14}O_7S$	337.0391	337.0387	-1.2	**257**; 151	DA-O-Sul	nd	LQ; D^L	nd
$23^{\Delta\#}$	17.30	264	$C_{15}H_{12}O_7S$	335.0239	335.0231	-2.3	**255**	LG-O-Sul	D^L	LQ; LG; LA; ILQ; D^L	nd
$24^{*\#}$	17.84	364	$C_{26}H_{30}O_{13}$	549.1617	549.1614	-0.6	**255**; 417	ILA	LA; ILA	LA; ILA; D^L	ILA
25^{Δ}	17.92	366	$C_{21}H_{20}O_{10}$	431.0990	431.0984	1.5	**255**; 175	ILG-O-GluA	ILG; D^L	ILG	nd
$26^{*\#}$	18.88	364	$C_{21}H_{22}O_9$	417.1194	417.1191	-0.6	**255**; 297	ILQ	LQ; LA; ILA; ILQ; D^L	LQ; ILQ	nd
27^{*}	19.30	330	$C_{15}H_{10}O_4$	253.0501	253.0506	2.2	135	DHF	D^L	DHF; D^L	DHF
28^{Δ}	19.63	/	$C_{21}H_{22}O_{10}$	433.1147	433.1140	-1.6	**257**	DA-O-GluA	D^L	LG; LA; D^L	nd
$29^{\Delta\#}$	19.87	226	$C_{15}H_{14}O_6S$	321.0443	321.0438	-1.3	**241**	Equol-O-Sul	D^L	ONO; D^L	nd
$30^{\Delta\#}$	19.88	266	$C_{22}H_{20}O_{10}$	443.0982	443.0984	0.4	**267**; 252	FOR-O-GluA	ONO; D^L	D^L	nd

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31$^{\Delta\#}$	20.24	274	$C_{21}H_{22}O_{10}$	433.1150	433.1140	-2.3	**257**; 175; 151	DA-*O*-GluA	D^L	LQ; LG; LA; ILQ;	nd
										ILG; ILA; D^L	
32$^{\Delta}$	20.4	/	$C_{15}H_{14}O_7S$	337.0396	337.0387	-2.5	**257**	*O*-Demethylangolensin-*O*-S	nd	ONO	nd
								ul			
33$^{\Delta\#}$	20.47	376	$C_{21}H_{20}O_{10}$	431.0980	431.0984	0.9	**255**; 175	ILG-*O*-GluA	LQ; LG; LA;	LQ; LG; ILQ;	nd
									ILQ; ILG; D^L	ILG; D^L	
34$^{*\#}$	20.84	248	$C_{15}H_{10}O_4$	253.0511	253.0506	-1.8	**253**	DZ	ONO; D^L	ONO; D^L	ONO
35	21.87	/	$C_{16}H_{12}O_5$	283.0609	283.0612	0.9	**268**	FOR monohydroxylate	nd	ONO; D^L	nd
36$^{*\#}$	22.55	276	$C_{15}H_{12}O_4$	255.0666	255.0663	-1.4	**135**; 153; 119	LG	LG; ILG; D^L	LQ; LA; ILQ; LG;	LG;
										D^L	ILG;
											LQ; LA
37$^{\#}$	22.84	/	$C_{16}H_{12}O_5$	283.0616	283.0612	-1.4	**268**; 251	FOR monohydroxylate	nd	ONO; D^L	nd
38$^{\Delta\#}$	23.53	274	$C_{15}H_{14}O_7S$	337.0390	337.0387	-0.8	**257**; 151	DA-*O*-Sul	D^L	LQ; LG; LA; ILQ;	nd
										ILG; ILA; D^L	

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39[#]	24.26	258	$C_{16}H_{12}O_5$	283.0615	283.0612	-1.2	**268**; 245	FOR monohydroxylate	nd	ONO; D^L	nd
40[Δ#]	25.59	262	$C_{15}H_{14}O_7S$	337.0388	337.0387	-0.2	**257**; 151	DA-O-Sul	D^L	LQ; LG; LA; ILQ; ILG; ILA; D^L	nd
41[Δ#]	27.22	372	$C_{15}H_{12}O_7S$	335.0235	335.0231	-1.2	**255**	ILG-O-Sul	ILG; LG; LA; D^L	LQ; LG; ILQ; ILG; D^L	nd
42[Δ#]	28.10	282	$C_{16}H_{12}O_7S$	347.0236	347.0231	-1.5	**267**	FOR-O-Sul	ONO	ONO; D^L	nd
43[#]	28.23	/	$C_{16}H_{12}O_5$	283.0615	283.0612	-1.2	**268**	FOR monohydroxylate	nd	ONO; D^L	nd
44[*#]	30.82	278	$C_{15}H_{14}O_4$	257.0824	257.0819	-1.6	**151**	DA	nd	LQ; LA; ILQ; ILA; D^L	LQ; LA; ILG
45[#]	30.84	/	$C_{15}H_{14}O_4$	257.0818	257.0819	0.5	**151**	O-Demethylangolensin	ONO; D^L	ONO	nd
46[*#]	32.42	372	$C_{15}H_{12}O_4$	255.0665	255.0663	-0.9	**135**; 153; 119	ILG	LG; ILQ; ILG; D^L	LG; LQ; LA; ILQ; ILG; D^L	nd
47[*#]	33.04	250	$C_{16}H_{12}O_4$	267.0663	267.0663	-0.4	**252**	FOR	ONO; D^L	ONO; D^L	ONO
48[*#]	33.29	250	$C_{42}H_{61}O_{16}$	821.3969	821.3965	-0.5	**351**; 645	GLY	GLY; D^L	GLY; D^L	GLY

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49[#]	33.82	269	$C_{16}H_{14}O_4$	269.0816	269.0819	1.2	**254**	Dihydroformononetin	nd	ONO; D[L]	nd
50[Δ]	34.04	/	$C_{37}H_{42}O_{18}$	773.2318	773.2298	-2.5	**597**; 421	IAA-di-O-GluA	IAA	IAA	nd
51	36.12	/	$C_{25}H_{26}O_8$	453.1568	453.1555	-2.9	395	IAA dihydroxylate	nd	IAA	nd
52[Δ]	38.34	/	$C_{37}H_{42}O_{18}$	773.2290	773.2298	1.8	**597**; 421	IAA-di-O-GluA	IAA	IAA	nd
53	38.86	/	$C_{25}H_{26}O_8$	453.1570	453.1555	-3.2	**381**; 435; 353	IAA dihydroxylate	nd	IAA	nd
54	39.65	/	$C_{25}H_{26}O_8$	453.1563	453.1555	-1.8	**381**; 435; 353	IAA dihydroxylate	nd	IAA; D[L]	nd
55[Δ]	42.80	270	$C_{31}H_{34}O_{12}$	597.1966	597.1978	2.0	**421**	IAA-O-GluA	nd	IAA	nd
56	42.87	268	$C_{25}H_{26}O_7$	437.1616	437.1606	-2.3	419; 379	IAA monohydroxylate	nd	IAA	nd
57	42.91	/	$C_{36}H_{54}O_{10}$	645.3652	645.3644	-1.2	**469**	Glycyrrhetinic acid-O-GluA	GLY	nd	GLY
58	45.56	268	$C_{25}H_{26}O_7$	437.1612	437.1606	-1.4	407; 368	IAA monohydroxylate	nd	IAA	IAA
59	45.63	270	$C_{25}H_{24}O_9$	467.1356	467.1348	-1.8	449; 409	IAA aldehyde derivative	IAA	IAA	nd
60[#]	45.47	/	$C_{36}H_{54}O_{10}$	645.3649	645.3644	-0.5	**469**	Glycyrrhetinic acid-O-GluA	GLY; D[L]	nd	GLY
61	46.38	270	$C_{25}H_{26}O_7$	437.1620	437.1606	-3.3	365	IAA monohydroxylate	IAA	IAA; D[L]	nd
62	46.59	288	$C_{25}H_{26}O_9$	469.1529	469.1504	-5.3	451; 419	IAA trihydroxylate	nd	IAA	nd

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63[#]	46.69	/	$C_{30}H_{46}O_5$	485.3271	485.3272	0.2	**441**; 371	Hydroxyglycyrrhetinic acid	GLY; D[L]	nd	GLY
64	46.77	270	$C_{25}H_{26}O_9$	469.1521	469.1504	-3.6	**437**; 421; 367	IAA trihydroxylate	nd	IAA	nd
65[Δ]	47.27	/	$C_{31}H_{34}O_{12}$	597.1951	597.1978	4.4	**421**	IAA-*O*-GluA	IAA	IAA	nd
66[#]	47.92	270	$C_{25}H_{26}O_7$	437.1621	437.1606	-3.5	382; 235	IAA monohydroxylate	nd	IAA	nd
67[*#]	50.24	234	$C_{25}H_{26}O_6$	421.1661	421.1657	-1.1	352	IAA	IAA	IAA; D[L]	IAA
68[*#]	51.98	250	$C_{30}H_{46}O_4$	469.3324	469.3323	-0.2	**425**	Glycyrrhetinic acid	GLY; D[L]	nd	GLY

[Δ], Confirmed by enzyme hydrolysis; [*], Confirmed by comparing with reference standards; [#], Detected in LWE (licorice water extract) high dosage bio-samples by LC/MS; D[L], Detected in LWE normal dosage bio-samples by LC/SRM-MS. **Bold**, base peak. GluA, glucuronide; Sul, sulfate; DA, Davidigeinin; DZ, Daidzein; DHF, 7,4′-dihydroxyflavone; FOR, Formononetin; GLY, Glycyrrhizic acid; IAA, Isoangustone A; ILG, Isoliquiritigenin; ILQ, Isoliquiritin; ILA, Isoliquiritin apioside; LG, Liquiritigenin; LQ, Liquiritin; LA, Liquiritin apioside; ONO, ononin.

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Table 2. Characterization of licorice metabolites in rats after oral administration of high dosage (20 g/kg) of licorice water extract by HPLC-DAD-ESI-MS^n and LC/qTOF-MS (metabolites characterized in Table 1 not included).

Deriving licorice compound (MW)	No.	RT	λ$_{max}$ (nm)	Formula	[M-H]$^-$ Measured	[M-H]$^-$ Predicted	Diff. (ppm)	MS/MS	Metabolite	Plasma	Urine
Choerospondin (434)	69[a]	10.34	/	$C_{21}H_{22}O_{14}S$	529.0652	529.0657	1.0	**353**, 449	Naringenin dihydrochalcone-O-GluA-O-Sul	nd	D^L; D^H
	70[a]	11.74	/	$C_{21}H_{20}O_{14}S$	527.0504	527.0501	-0.6	**351**, 447	Naringenin-O-GluA-O-Sul	D^H	D^H; D^L
	71[a]	16.21	284	$C_{21}H_{20}O_{11}$	447.0943	447.0933	-2.2	**271**, 175	Naringenin-O-GluA	D^H	D^H; D^L
	72[a]	16.85	/	$C_{21}H_{20}O_{11}$	447.0949	447.0933	-3.7	**271**, 175	Naringenin-O-GluA	nd	D^H; D^L
	73[a]	17.39	/	$C_{21}H_{22}O_{11}$	449.1097	449.1089	-1.7	**273**	Naringenin dihydrochalcone-O-GluA	D^L	D^H; D^L
	74[a]	18.64	/	$C_{21}H_{22}O_{11}$	449.1091	449.1089	-0.4	**273**	Naringenin dihydrochalcone-O-GluA	nd	D^H; D^L
	75[a]	19.68	284	$C_{15}H_{14}O_8S$	353.0344	353.0337	-2.0	**273**, 151	Naringenin dihydrochalcone-O-Sul	nd	D^H; D^L
	76[a]	25.83	/	$C_{15}H_{12}O_8S$	351.0184	351.0180	-1.1	**271**	Naringenin-O-Sul	D^H; D^L	D^L
Cyclolicoflavanone	77[a]	31.80	/	$C_{26}H_{28}O_{11}$	515.1557	515.1559	0.4	**339**, 175	Cyclolicoflavanone-O-GluA	nd	D^H; D^L

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(340)

	78[a]	32.51	/	$C_{26}H_{28}O_{11}$	515.1562	515.1559	-0.6	**339**, 175	Cyclolicoflavanoe-O-GluA	D^L	D^H; D^L
Genkwanin (284)	**79**[a]	17.44	312	$C_{22}H_{20}O_{14}S$	539.0504	539.0501	-2.6	**459**, 357	Genkwanin-O-GluA-O-Sul	D^H	nd
Glycycoumarin	**80**[a]	24.07	/	$C_{33}H_{36}O_{18}$	719.1853	719.1829	-3.4	**367**, 352	Glycycoumarin-di-O-GluA	D^H	D^L
(368)											
	81[a]	24.14	/	$C_{27}H_{28}O_{12}$	543.1517	543.1508	-1.7	**367**, 497	Glycycoumarin-O-GluA	D^H; D^L	D^H; D^L
	82[a]	25.09	/	$C_{27}H_{28}O_{12}$	543.1507	543.1508	0.3	**367**, 497	Glycycoumarin-O-GluA	D^H; D^L	D^H; D^L
Licoisoflavone A	**83**[a]	23.21	/	$C_{32}H_{34}O_{18}$	705.1691	705.1672	-2.6	**529**, 353	Licoisoflavone A-di-O-GluA	D^H	D^H; D^L
(354)											
	84[a]	24.20	260	$C_{32}H_{34}O_{18}$	705.1693	705.1672	-2.9	**529**,658,353	Licoisoflavone A-di-O-GluA	D^H	D^H; D^L
	85[a]	31.20	280	$C_{26}H_{26}O_{12}$	529.1359	529.1351	-1.5	**353**, 175	Licoisoflavone A-O-GluA	D^L	D^H; D^L
	86[a]	33.62	/	$C_{26}H_{26}O_{12}$	529.1362	529.1351	-2.0	**353**, 175	Licoisoflavone A-O-GluA	D^H; D^L	D^H
Licoisoflavone B	**87**[a]	34.96	262	$C_{26}H_{24}O_{12}$	527.1206	527.1195	-0.4	**351**	Licoisoflavone B-O-GluA	D^L	D^H; D^L
(352)											

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Licorice-saponin A3 (984)	**88**	25.42	/	$C_{48}H_{72}O_{21}$	983.4488	983.4493	0.5	**821**, 645, 351	Licorice-saponin A3	D^H	nd
Licorice-saponin G2 (838)	**89**	30.76	/	$C_{42}H_{62}O_{17}$	837.3903	837.3914	0.5	**351**, 661, 776	Licorice-saponin G2	D^H	nd
Semilicoisoflavone B (352)	**90**[△]	25.55	260	$C_{27}H_{28}O_{11}$	527.1202	527.1195	-1.3	**351**, 175	Semilicoisoflavone B-*O*-GluA	D^H	D^H; D^L

[△], Confirmed by enzyme hydrolysis; **Bold**, base peak; GluA, glucuronide; Sul, sulfate; D^H, Detected in LWE (licorice water extract) high dosage bio-samples by LC/MS; D^L, Detected in LWE normal dosage bio-samples by LC/SRM-MS.

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Scheme 1


```
                    Traditional Chinese Medicine
                               ↓
                       Chemical fingerprint
                          ↓           ↓

Metabolism of single compounds    Identify more metabolites at a high dosage

  HPLC/DAD/ESI-MSⁿ and              HPLC/DAD/ESI-MSⁿ and
  LC/qTOF-MS analysis               LC/qTOF-MS analysis
         ↓                                  ↓
  UV and MS spectral data           LC/MS profiles and spectral data
      ↓        ↓                          ↓            ↓
 Comparison   Enzyme           Comparison with      Comparison with
 with         hydrolysis       blank samples        fingerprint
 standards                          ↓                    ↓
      ↓        ↓                 Enzyme    ↔      TCM compounds
 characterization of metabolites  hydrolysis        database
             ↓                      ↓                ↓
    Summarization of            Metabolite structures
    metabolic pathways

 In vivo metabolic profile of TCM at a normal dosage

  Structural information for possible metabolites
                     ↓
               SRM ion pairs
                     ↓
          establish LC/SRM-MS method
                     ↓
     Analysis of bio-samples at a normal dosage
                     ↓
       Identification of TCM in vivo metabolites
```

Scheme 2

LA (**10**)

ILA (**24**)

6

LQ (**12**)

ILQ (**26**)

1

11, 13

21, 23

LG (**36**)

ILG (**46**)

3, 7

18, 25, 33

41

19

15, 28, 31

Davidigenin (**44**)

9, 22, 38, 40

16, 20

Scheme 3

Formononetin-*O*-GluA (**30**)

Formononetin-*O*-Sul (**42**)

Formononetin (**47**)

Dihydroformononetin (**49**)

Formononetin monohydroxylate
(**35**, **37**, **39**, **43**)

Daidzein-di-*O*-Sul (**2**, **4**)

Daidzein (**34**)

Equol-*O*-Sul (**29**)

Daidzein-*O*-Sul (**14,17**)

O-Demethylangolensin (**45**)

O-Demethylangolensin-*O*-Sul (**32**)

Scheme 4

1: Plasma

XIC: m/z 447

71

m/z 447→271, 175
271→151
λ_{max}: 284 nm

Enzyme hydrolysis →

4: Aglycone

Naringenin

2: LWE

XIC: m/z 271

m/z 433→271
271→151
λ_{max}: 290 nm
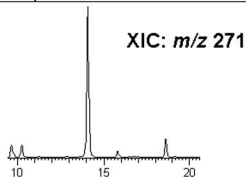
5: Deriving compound
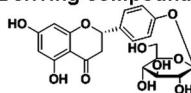
Choerospondin
MW 434

3: Licorice compounds database

Choerospondin (naringenin-4'-O-glucoside)
MW: 434
λ_{max}: 290 nm

Metabolic pathway

6: More metabolites

Glucuronides: 71, 72
Sulfate: 76
Glucuronide-sulfate: 70
Dihydrochalcones: 69, 73, 74, 75

Figure 1
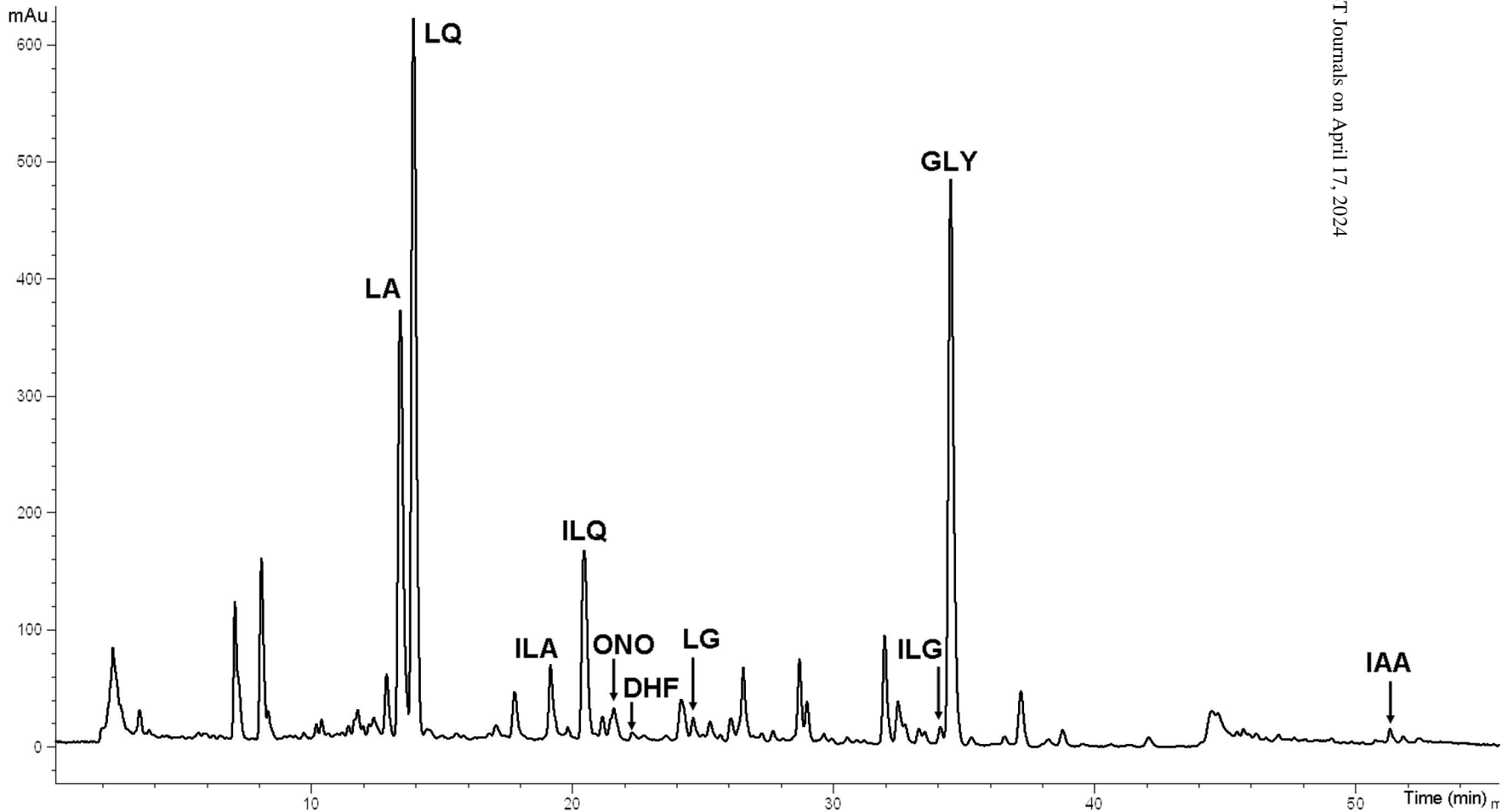
Figure 2
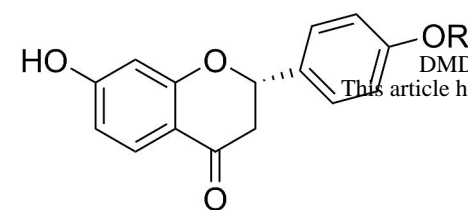
LA (**10**) R=Glc-*O*-Api
LQ (**12**) R=Glc
LG (**36**) R=H
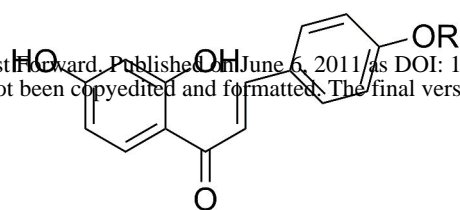
ILA (**24**) R=Glc-*O*-Api
ILQ (**26**) R=Glc
ILG (**46**) R=H
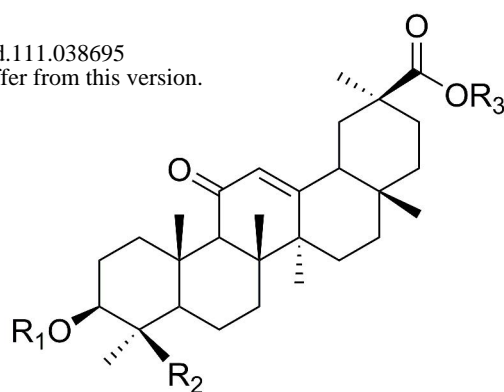
GLY (**48**) R_1=GluA(2,1)-*O*-GluA, R_2=R_3=H

Glycyrrhetinic acid (**68**) R_1=R_2=R_3=H

Licorice-saponin A3 (**88**) R_1=GluA(2,1)-*O*-GluA, R_2=H, R_3=Glc

Licorice-saponin G2 (**89**) R_1=GluA(2,1)-*O*-GluA, R_2=CH_2OH, R_3=H
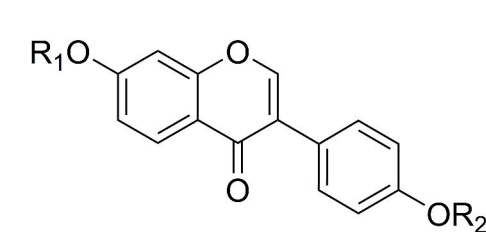
Daidzein (**34**) R_1=R_2=H
Formononetin (**47**) R_1=H, R_2=CH_3
ONO R_1=Glc, R_2=CH_3
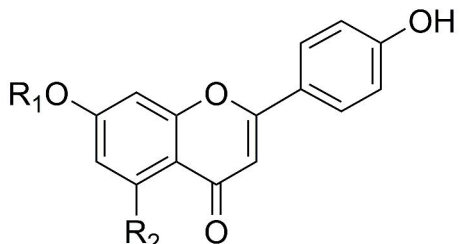
DHF (**27**) R_1=H, R_2=H
Genkwanin R_1=OCH_3, R_2=OH
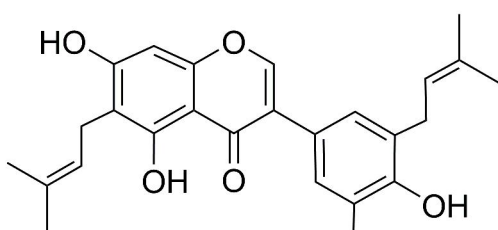
IAA (**67**)
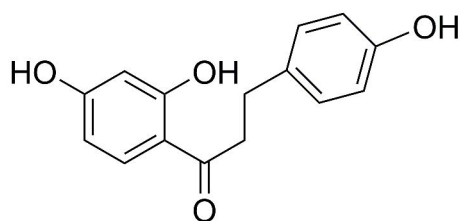
Davidigenin (**44**)
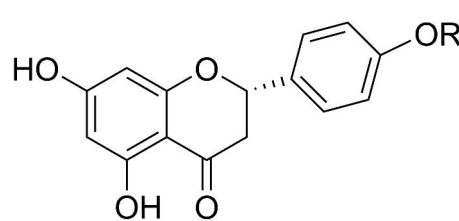
Naringenin R=H
Choerospondin R=Glc
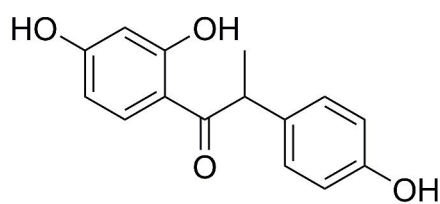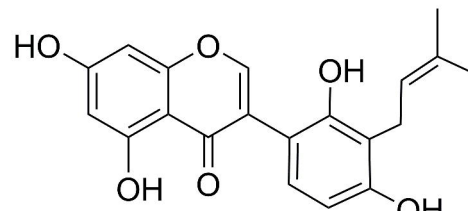
O-Demethylangolensin (**45**)
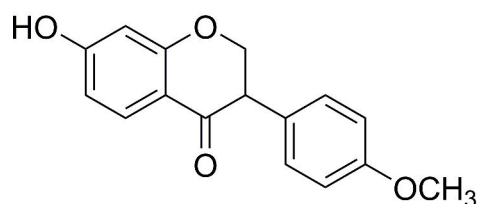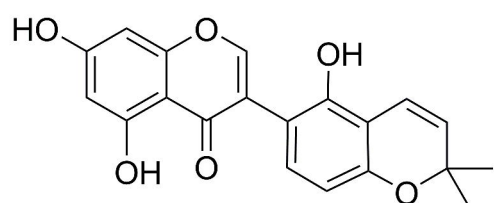
Dihydroformononetin (**49**)
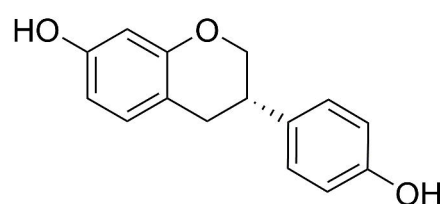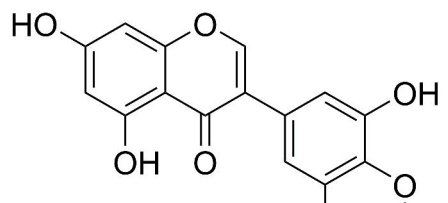
S-Equol
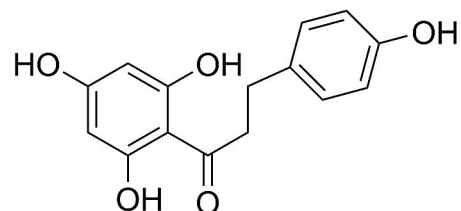
Naringenin dihydrochalcone
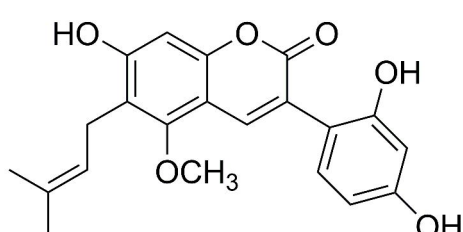
Glycycoumarin
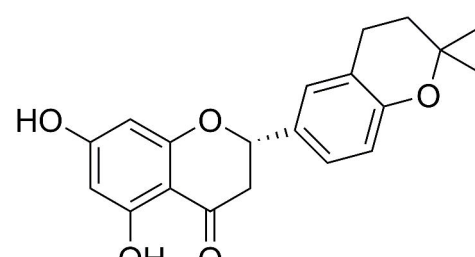
Licoisoflavone A

Licoisoflavone B

Semilicoisoflavone B

Cyclolicoflavanone

Figure 3