

Absorption and metabolism of Astragali Radix decoction:

in silico, in vitro and a case study in vivo

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Abbreviations: HPLC-DAD-ESI-MSⁿ, high-performance liquid chromatography-diode array detection-electrospray ion trap tandem mass spectrometry; UV, ultraviolet; ClogP, the LogP value calculated by ClogP 4.0 program, P is partition coefficient of the molecule; C1: calycosin-7-*O*- β -D-glucuronide; C2: (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucuronide; C3: 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-2'-*O*- β -D-glucuronide; C4: 7,2'-dihydroxy-3',4',5'-trimethoxyisoflavan-7-*O*- β -D-glucuronide; C5: 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucuronide; C6: (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside-6''-*O*-malonate; C7: 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside-6''-*O*-malonate; C8: calycosin-7-*O*- β -D-glucoside; C9: formononetin-7-*O*- β -D-glucoside; C10: (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-sambubioside; C11: (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpane-3-*O*- β -D-glucoside; C12: 7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside; C13: calycosin; C14: formononetin; C15: (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan; C16: 7,2'-dihydroxy-3',4'-dimethoxyisoflavan; C17: formononetin-7-*O*- β -D-glucuronide; C18: calycosin sulphate; C19: 7,2'-dihydroxy-3',4',6'-trimethoxyisoflavan; C20: daidzein-7-*O*- β -D-glucuronide; C21: 7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7-*O*- β -D-glucosyl-3'-*O*- β -D-glucuronide

Abstract

To profile absorption of Astragali Radix decoction and identify its oral absorbable constituents and their metabolites, four complementary *in silico*, *in vitro* and *in vivo* methods, *i.e.*, computational chemistry prediction method, Caco-2 cell monolayer model experiment, improved rat everted gut sac experiment and healthy human volunteer experiment were used. According to *in silico* computation result, 26 compounds of Astragali Radix could be regarded as oral available compounds, including 12 flavonoids. In the *in vitro* and *in vivo* experiments, 21 compounds were tentatively identified by high-performance liquid chromatography-diode array detection-electrospray ion trap tandem mass spectrometry data, which involved calycosin, formononetin, (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan, 7,2'-dihydroxy-3',4'-dimethoxyisoflavan, calycosin-7-*O*- β -D-glucoside, formononetin-7-*O*- β -D-glucoside, 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside-6''-*O*-malonate, (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside and phase II metabolites calycosin-7-*O*- β -D-glucuronide, formononetin-7-*O*- β -D-glucuronide, (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucuronide, 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucuronide and calycosin sulphate. Calycosin and formononetin were proved absorbable by four methods; (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan and 7,2'-dihydroxy-3',4'-dimethoxyisoflavan were proved absorbable by three methods; formononetin-7-*O*- β -D-glucoside and

(6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpane-3-*O*- β -D-glucoside were proved absorbable by two methods. The existence of calycosin-7-*O*- β -D-glucuronide, formononetin-7-*O*- β -D-glucuronide, (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucuronide, 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucuronide and calycosin sulphate was proved by two or three methods. We found that besides isoflavones, pterocarpanes and isoflavans also could be metabolized by the intestine during absorption, and the major metabolites were glucuronides. In conclusion, the present study demonstrated that the flavonoids in Astragali Radix decoction, including isoflavones, pterocarpanes and isoflavans, could be absorbed and metabolized by intestine. These absorbable compounds, which were reported to have various bioactivities related to the curative effects of Astragali Radix decoction, could be regarded as an important component part of the effective constituents of Astragali Radix decoction.

Astragali Radix, a commonly used traditional Chinese drug, which is called Huangqi in Chinese, is derived from the dried roots of *Astragalus membranaceus* (Fisch.) Bunge or *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao (The Pharmacopoeia Commission of People's Republic of China, 1997). It has been used as a *qi*-tonifying drug in China for about 2000 years (first recorded in Shennong Bencao Jing, a materia medica book edited in 1st century). In general, its traditional usage is to be prepared as a decoction alone or to be prepared as a decoction together with other crude drugs (such as Chuanxiong Rhizoma, Angelica sinensis Radix) for oral administration.

Pharmacological studies indicate that Astragali Radix has various bioactivities, such as hypotensive (Hikino et al., 1976), inducing vasodilatation (Zhang et al., 2005), antioxidative (Shirataki et al., 1997), immunostimulating (Lee et al., 2003), antiviral (Anonymous, 2003), inducing cancer cell apoptosis (Cheng et al., 2004), reducing the capillary hyperpermeability and alleviating the dyskinesia caused by cerebral ischemia (Quan and Du, 1998), inhibiting cyclooxygenase-2 (Kim et al., 2001), promoting the motility of human spermatozoa (Liu et al., 2004), enhancing cardiovascular function, protecting the myocardium in diabetic nephropathy (Chen et al., 2001), anti-aging (Wang et al., 2003), hepatoprotective effect (Zhu et al., 2001), inhibiting sterol biosynthesis (Sung, 1999) and antibacterial (Hu et al., 2005).

Clinical researches show that Astragali Radix can improve cardiovascular function, restore and strengthen immune response, and enhance vitality. Indications supported by clinical trials include acute myocardial infarction, impaired immunity, viral

infections and adjunctive cancer treatment (Anonymous, 2003).

Regarding the chemical constituents of Astragali Radix, more than 100 compounds have been isolated and identified up to now, such as flavonoids (Subarnas et al., 1991; Lin et al., 2000), triterpene saponins (Kitagawa et al., 1983), polysaccharides, amino acids, phytosterols and phenolic acids.

As mentioned above, many pharmacological, clinical and phytochemical investigations on Astragali Radix have been conducted so far. However, researchers still don't know what its effective constituents are, how many compounds are absorbed into our blood after oral administration of the decoction, and what the fate of the decoction in our body is. The reasons include: **(i)** in clinical studies, the materials to be tested are usually the whole prescription or its different solvent extracts, and the researchers are always not clear about the chemical compositions of them; **(ii)** in phytochemical researches, the amounts of compounds isolated are always so small that it is impossible to carry out *in vivo* pharmacological or clinical experiments; **(iii)** although many researchers have ascribed immunomodulating activity to polysaccharides, antioxidative action to flavonoids and triterpene saponins, and hypotensive to γ -aminobutyric acid, these conclusions were mainly drawn from *in vitro* pharmacological experiments or the compounds investigated were always administrated by injection instead of per oral; **(iv)** no research on the intestinal absorption and metabolism of Astragali Radix decoction has been conducted to date. As a result, the knowledge about the absorption and metabolism of Astragali Radix decoction is very poor, and it is hard to correlate the compounds isolated from

Astragali Radix with the curative effects of Astragali Radix decoction.

In the present work, our aim was to profile the absorption of Astragali Radix decoction and identify its absorbable constituents and their metabolites.

We established a chemical database containing 124 compounds of Astragali Radix for estimating the drug-like properties of these compounds and predicting their oral absorption properties.

Two *in vitro* models, namely Caco-2 cell monolayer model and improved rat everted gut sac model, were used to profile the absorption of Astragali Radix decoction, with the aid of high-performance liquid chromatography-diode array detection-electrospray ion trap tandem mass spectrometry (HPLC-DAD-ESI-MSⁿ)¹.

The absorbable compounds and their metabolites in the urine samples of a healthy male volunteer orally dosed Astragali Radix decoction were also identified.

It was found that the flavonoids in Astragali Radix decoction, including isoflavones, pterocarpan and isoflavans, could be absorbed and metabolized by intestine and their main metabolites were glucuronides. They could be regarded as an important component part of the effective constituents of Astragali Radix decoction.

Materials and Methods

Materials and Chemicals. Astragali Radix was purchased from Hunyuan County in Shanxi Province of China, which was authenticated by Prof. Shaoqing Cai as the dried roots of *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao), and its voucher specimen (No. 2688) was deposited in the Herbarium of Pharmacognosy, School of Pharmaceutical Sciences, Peking University Health Science Center. Calycosin and calycosin-7-*O*- β -D-glucoside (purity>95%) were isolated from the roots of *Astragalus membranaceus* var. *mongholicus* by one of the author, Prof. Pengfei Tu. HPLC grade acetonitrile was purchased from Fisher Scientific (Loughborough, UK). Pure water was purchased from Hangzhou Wahaha Group Co., Ltd. (Hangzhou, China). NaHCO₃ and NaCl of analytical grade were obtained from Beijing Beihua Fine Chemicals Co., Ltd. (Beijing, China). Medium 199 powder (GibcoTM, with Earle's salts and L-glutamine, without NaHCO₃, Category No. 31100035) was purchased from Invitrogen Corp. (Carlsbad, California, USA). Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Hanks' balanced salt solution, Earle's balanced salt solution, fetal calf serum and other culture media and supplements were obtained from Gibco (Grand Island, NY, USA). Transwells were purchased from Corning Costar (Cambridge, MA, USA).

Instrumentation. An Agilent 1100 LC-MSD-Trip-SL system (Agilent Technologies, Palo Alto, CA, USA) consists of a degasser, an autosampler, a column thermostat, a quaternary pump, a diode-array detector and an electrospray ion trap mass spectrometer. The column used was a Zorbax SB-C₁₈ (4.6 × 250mm, 5 μ m)

HPLC column with a Zorbax SB-C₁₈ (4.6 × 12.5 mm, 5 μm) guard column (Agilent Technologies, Palo Alto, CA, USA).

Construction of the chemical database of Astragali Radix. The database was created by us using ChemFinder Ultra 8.0 (CambridgeSoft Corporation, Cambridge, MA, USA) under Microsoft® Windows® 2000 Professional system (Microsoft Corporation China Ltd., Beijing, China). The database contains one table with 26 searchable fields, including Structure, Molecular identification, Formula, Molecular weight, Accurate mass, Name, Chinese name, Original plant, Physical properties, Chemical abstracts service registry number, Simplified molecular input line entry specification, ClogP, Topological molecular polar surface area, Number of hydrogen bond acceptors, Number of hydrogen bond donors, Number of rotatable bonds, Number of violations of “rule of 5”, UV, Mass spectrum data, Infrared data, ¹H-nuclear magnetic resonance data, ¹³C-nuclear magnetic resonance data, References, Biological activities, Biological activities references and Notes. Records of 124 compounds were inputted into the database according to the phytochemical and pharmacological literatures of Astragali Radix and the Combined Chemical Dictionary on CD-ROM version 8.1 (Chapman & Hall/CRC, FL, USA). Molecular weight and accurate mass were calculated by ChemDraw Ultra 8.0 (CambridgeSoft Corporation, Cambridge, MA, USA). ClogP was calculated by ClogP4.0 program (BioByte Corporation, Claremont, CA, USA). The number of hydrogen bond acceptors, the number of hydrogen bond donors, the number of violations of “rule of 5”, topological molecular polar surface area and the number of rotatable bonds were

calculated by the free Molinspiration Property Calculation Services on the Internet (<http://www.molinspiration.com/cgi-bin/properties>).

***In silico* prediction of oral absorption properties of Astragali Radix constituents.** Two simple counting methods were used to predict oral absorption properties of Astragali Radix constituents. Firstly, the “rule of 5” descriptors (Lipinski et al., 1997), *i.e.*, molecular weight, ClogP, the number of hydrogen bond acceptors, the number of hydrogen bond donors, were used to estimate oral absorption properties of the constituents. The molecules which met the “rule of 5” were considered as good oral absorbable compounds. Secondly, the molecules which contained 10 or fewer rotatable bonds and topological molecular polar surface area (Ertl et al., 2000) of which were equal to or less than 140 \AA^2 were regarded as good oral absorbable compounds (Veber et al., 2002). The “rule of 5” set a lower limit of molecular polarity, and the rule that the number of rotatable bonds ≤ 10 and topological molecular polar surface area $\leq 140 \text{ \AA}^2$ set an upper limit of molecular polarity. Therefore, we considered that the compounds met both rules were oral available compounds.

Preparation of freeze-drying powder of Astragali Radix decoction. Astragali Radix was cut into decoction pieces about 1 cm long. Two hundred grams decoction pieces were weighed and soaked with 2.0 L water for 30 min. Then, the decoction pieces were boiled for 30 min and the decoction was filtrated out by absorbent cotton inserted in a funnel. Subsequently, the dregs were boiled twice again for 30 min with 1.6 L and 1.2 L water successively, and the decoctions were filtrated out with the above method. Afterwards, the decoctions of three times were merged and condensed

by a Heidolph Laborota 4001 rotatory evaporator (Heidolph Instruments GmbH&Co., Schwabach, Germany) under reduced pressure. Finally, the concentrate was lyophilized to 70.6 g powder by a Labconco Freezone[®] 6 freeze dryer (Labconco Corporation, Kansas, MO, USA). Thus, each gram powder was equivalent to 2.83 g crude drug of Astragali Radix. The powder was stored in a desiccator at room temperature for later use.

Animals. Adult male Sprague-Dawley rats weighing between 250 to 340 g (The Experimental Animal Center of Peking University Health Science Center, Beijing, China) were used in the everted gut sac experiment. The animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health. The rats were fasted for 24 h before the date of the experiment.

***In vitro* improved rat everted gut sac experiment.** One package of Medium 199 powder (9.5 g) was dissolved in 1.00 L distilled water with the addition of 2.2 g NaHCO₃, then the Medium 199 solution was gassed with 95% O₂ + 5% CO₂ at 37°C. Twelve rats were euthanized by cervical dislocation, and the entire small intestine was quickly taken out and flushed three times by saline using a 20 ml syringe at room temperature. The intestine was instantly put in oxygenated Medium 199 solution. With the help of a smooth plastic rod (4.0 mm diameter), the intestine was gently everted as quickly as possible to make the serosal side towards the inside and the mucosal side towards the outside, and then the everted intestine was slid into the medium solution. Afterwards, one end of the intestine was tied with suture, and the

intestine was filled with 10 ml Medium 199 solution. Then, the intestine was sealed in the other end with suture (Barthe et al., 1998).

In control group, each of six everted gut sacs was put into an Erlenmeyer flask (250 ml) containing 100 ml pregassed (95%O₂ + 5%CO₂) Medium 199 solution at 37°C. In test group, each of six everted gut sacs was put into an Erlenmeyer flask (250 ml), which contained 100 ml pregassed (95%O₂ + 5%CO₂) Astragali Radix Medium 199 solution at 37°C with a concentration of 100 mg crude drug per milliliter.

The flasks of both test and control groups were plugged with rubber stoppers, which had two holes for gas (95%O₂ + 5%CO₂) in and out. Then, the sacs were incubated at 37°C in a DSY-2-4 type electroheating constant temperature water bath (Beijing Guohua Medical Apparatus and Instrument Factory, Beijing, China) for one hour, aerated by gas (95%O₂ + 5%CO₂). Afterwards the sacs were removed and blotted dry with gauze. Then the sacs were cut open and the serosal side solutions, which should contain absorbable constituents of Astragali Radix decoction or their metabolites, were drained into small tubes. At the same time, the mucosal side solutions which contained Astragali Radix decoction were also sampled for analysis. Both mucosal side and serosal side solutions were stored at -40°C in a MDF-U5410 Sanyo medical freezer (Sanyo Electric Co., Ltd, Osaka, Japan) until analyzed.

***In vitro* Caco-2 cell monolayer model experiment.** The cell monolayer was prepared as the method described previously (Yang et al., 2004). The monolayer with transepithelial electrical resistance values less than 800 ohms × cm² were not used. The monolayers were washed three times with Hanks' balanced salt solution, pH 7.4,

at 37 °C. In test group, an Astragali Radix Earle's balanced salt solution at a concentration of 100 mg crude drug per milliliter was added to the apical side. In control group, an Earle's balanced salt solution was added to the apical side. In both groups, Hanks' balanced salt solutions were added to the basolateral sides. Then, the samples were shaken (37°C, 50 rpm) and 200 µl aliquots were taken from the basolateral sides at 0, 45, 90, 135, 180 min. The apical solutions at 0 min were also sampled for analysis. All experiments were repeated three times. All of the apical solutions and basolateral solutions were stored at -40°C in a MDF-U5410 Sanyo medical freezer (Sanyo Electric Co., Ltd, Osaka, Japan) until analyzed.

***In vivo* human experiment.** In 10 days, the diet of a volunteer (a 28-year-old healthy Chinese male) was fixed throughout but water was allowed *ad libitum*. The diet didn't contain soybean, vegetables and fruits. The volunteer has three meals a day. Each meal consists of a piece of 400g bread which contains vitamin B₁, vitamin B₂, nicotinic acid, carotene, Zn, Fe and Ca (Mankattan Beijing Co., Ltd., Beijing, China), an egg and 20 g pure honey (Beijing Baihua Bee Product Co., Ltd., Beijing, China). The volunteer was prohibited from smoking and drinking alcoholic beverages. In day 3 and day 4, total volume urine samples were collected as blank urine samples. From day 5 to day 10, the volunteer took Astragali Radix decoction orally before meal at a dosage of 60 g crude drug twice a day, and the total volume urine samples were collected as drug-containing urine samples. This study was approved by ethics committee of Health Science Center of Peking University, and was carried out following good clinical practice guidelines and was in accordance with the guidelines

of the Declaration of Helsinki and all its amendments, and the subject had given his written consent.

Each of two blank urine samples and six drug-containing urine samples was evaporated to dryness under vacuum at 37°C by a Heidolph Laborota 4001 rotatory evaporator (Heidolph Instruments GmbH&Co., Schwabach, Germany). These eight dried samples were weighed by an electronic balance (Ohaus China, Shanghai, China), and 1.00 g of each was transferred to a centrifuge tube. Then the dried samples were extracted with 6ml methanol (HPLC grade, Fisher Scientific, Loughborough, UK) by sonication for 5 min using a TP-150 ultrasonic cleaner (Tianpong Electricity New Technology Co., Beijing, China). The extracts were centrifugated at 4800 rpm for 20 min using a Anke TDL-5-A centrifuge (Shanghai Anting Experimental Instrument Factory, Shanghai, China), and then the supernatant were applied to HPLC-DAD-ESI-MSⁿ analysis.

Sample analysis. All of the serosal, mucosal, apical, basolateral side solutions and uric samples were filtered through 0.45 µm micropore membranes (Tianjin Tengda Filter Factory, Tianjin, China) and were injected into the instrument for HPLC-DAD analysis directly. According to HPLC-DAD analytical results, representative samples were chosen. Then, representative samples and a calycosin and calycosin-7-*O*-β-D-glucoside methanol solution were further analyzed by HPLC-DAD-ESI-MSⁿ.

The samples were analyzed by a gradient method at a flow rate of 1.000 ml/min. The mobile phase consisted of water (A) and acetonitrile (B). The gradient was 0.0%

B at 0.00~5.00 min, 0.0~15.0% B at 5.00~20.00min, 15.0% B at 20.00~25.00 min, 20.0% B at 25.00~30.00 min, 20.0%~30.0% B at 30.00~35.00 min, 30.0% B at 35.00~40.00min, 30.0%~50.0% B at 40.00~50.00min, 50.0%~100.0% B at 50.00~60.00 min. The injection volume was 10.00~30.00 μ l. The column temperature was 30.0°C. In HPLC-DAD-ESI-MSⁿ analysis, HPLC chromatograms were recorded at 210, 230, 254, 280 and 365 nm, and UV and visible light spectra were obtained by scanning from 190 nm to 800 nm. The HPLC effluent was split, and about 200 μ l/min was introduced into the mass spectrometer.

The mass spectrometer was operated in alternating negative ion and positive ion electrospray mode and full scans were acquired from m/z 50 to 1500. The temperature of drying gas (N₂) was 325°C at a flow-rate of 7.00 L/min and a nebulizing pressure of 20.00 psi. A data-dependent program was used in the HPLC-DAD-ESI-MSⁿ analysis so that the two most abundant ions in each scan were selected and subjected to MS² and MS³ analyses. The collision-induced dissociation energy was varied automatically from 0.3eV to 2.0 eV in smart fragmentation mode. The isolation width of precursor ions was 4.0 mass units. All data were collected and processed by Agilent 1100 ChemStation 09.03 version and LC/MSD Trap Software 4.2 version (Agilent Technologies, Palo Alto, CA, USA).

Results

I. The Results of *in silico* Absorption Prediction, *in vitro* and *in vivo* Absorption Experiments of Astragali Radix Decoction

***In silico* prediction of oral available constituents of Astragali Radix.** Totally 124 compounds have been isolated from Astragali Radix (including *Astragalus membranaceus* (Fisch.) Bunge and *Astragalus membranaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao.) up to now (refer to Supplemental data – Table s1).

According to calculation results (refer to Supplemental data – Table s1), 68 compounds met the “rule of 5”, and 74 compounds met the rule that topological molecular polar surface area $\leq 140 \text{ \AA}^2$ and the number of rotatable bonds ≤ 10 . Sixty-two compounds met both rules.

Except 17 amino acids and 4 essential oil ingredients identified by gas chromatography-mass spectrometry, 41 compounds could be regarded as good oral available compounds. Among them, 26 compounds were isolated from *Astragalus membranaceus* var. *mongholicus*, including 12 flavonoids, 5 phenolic acids, 5 nitrogen-containing compounds, 3 lignanoids and 1 coumarin, and their structures were shown in Supplemental data – Fig. s1.

Absorption in the improved rat everted gut sac model. Fig. 1 shows the base peak chromatograms of the samples detected in negative ion mode. Eighteen peaks (C1~C18, denoting compound 1~18) were tentatively identified by HPLC-DAD-ESI-MSⁿ data. The peak areas of compound 1, 2, 3, 4 and 5 in the serosal side solution were larger than those of compound 1, 2, 3, 4 and 5 in the

mucosal side solution. This indicated that either compounds 1, 2, 3, 4, 5 might be metabolites or they might be absorbed by active transport. Besides, compound 17 and compound 18 were detected in both serosal side solution and mucosal side solution.

These identified compounds can be classified into three groups according to their retention time and structure type. **(i)** Four flavonoid aglycons: calycosin (C13); formononetin (C14); (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan (C15) and 7,2'-dihydroxy-3',4'-dimethoxyisoflavan (C16). **(ii)** Seven flavonoid glycosides: calycosin-7-*O*- β -D-glucoside (C8); formononetin-7-*O*- β -D-glucoside (C9); (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside (C11); (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-sambubioside (C10); (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside-6''-*O*-malonate (C6); 7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside (C12) and 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside-6''-*O*-malonate (C7). **(iii)** Seven flavonoid metabolites: calycosin-7-*O*- β -D-glucuronide (C1); calycosin sulphate (C18); formononetin-7-*O*- β -D-glucuronide (C17); (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucuronide (C2); 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-2'-*O*- β -D-glucuronide (C3); 7,2'-dihydroxy-3',4',5'-trimethoxyisoflavan-7-*O*- β -D-glucuronide (C4) and 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucuronide (C5).

Absorption in the Caco-2 monolayer model. Fig. 2 shows the base peak chromatograms of the samples detected in negative ion mode. Six peaks (C18, C13, C14, C19, C15, C16) were tentatively identified by HPLC-DAD-ESI-MSⁿ data. The

peak area of C18 in basolateral solution was larger than that in apical solution. This indicated that either C18 might be a metabolite or it might be absorbed by active transport. Five of these compounds (C13, C14, C15, C16, C18) were identical with those identified in improved rat everted gut sac experiment. C19 was identified as 7,2'-dihydroxy-3',4',6'-trimethoxyisoflavan.

Absorption and metabolism in human. The extracted ion chromatograms at m/z 363, 475, 477, 443, 459, 429, 639, 283, 267 of blank urine and drug-containing urine detected in negative ion mode are shown in Fig. 3. Seven peaks (C20, C1, C17, C21, C18, C2, C5) were tentatively identified by HPLC-DAD-ESI-MSⁿ data. Besides these seven compounds, calycosin (C13) and formononetin (C14) were also detected and identified by their HPLC retention times and UV spectra. In nine compounds, seven compounds (C1, C2, C5, C13, C14, C17, C18) were identical with those identified in rat everted gut sac experiment. The others were identified as daidzein-7-*O*- β -D-glucuronide (C20) and 7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7-*O*- β -D-glucosyl-3'-*O*- β -D-glucuronide (C21).

II. The Results of Identification of Absorbable Compounds and Their Metabolites.

The molecular weight of a compound in our study was confirmed by its molecular ion, quasi-molecular ion, dimer ion and adduct ion in negative and positive ion mass spectra. Then, we searched for this molecular weight in the chemical database of Astragali Radix and in the Combined Chemical Dictionary on CD-ROM version 8.1

to find relevant compounds. The structure type of the compound was judged by its UV spectrum. The structure of the compound was elucidated based on its MS² and MS³ data, and when possible, by direct comparison with the data of standard compounds and the data in the literatures (Lin et al., 2000; Xiao et al., 2004). As for metabolites, we first confirmed their structure types by MS² and MS³ data, and then identified their aglycons by above-mentioned method. Altogether 21 compounds were identified tentatively by HPLC-DAD-ESI-MSⁿ data, and their structures were shown in Fig. 4.

Identification of absorbable constituents of Astragali Radix decoction

Compound 13 (C13): C13 had a retention time of 42.1~42.9 min on HPLC. It showed [M-H]⁻ at m/z 283 in negative ion mass spectrum, and [M+H]⁺ at m/z 285, [M+Na]⁺ at m/z 307, [2M+Na]⁺ at m/z 591 in positive ion mass spectrum. So, its molecular weight was inferred to be 284 Da. Its UV spectrum exhibited maximum absorption at 200, 220, 250, 290 nm and shoulder peak at 310 nm with weak band I (310 nm) and strong band II (250 nm), which suggested that it was an isoflavone. The negative MS² spectrum of m/z 283 gave a fragment ion at m/z 268, and loss of 15 Da from the precursor ion indicated that there was a methyl in the molecule. In the chemical database of Astragali Radix, only calycosin was found to have a molecular weight of 284 Da. In addition, the HPLC retention time, UV spectrum, MSⁿ data of C13 were identical with those of standard compound of calycosin. So, C13 was identified as calycosin unequivocally.

Compound 14 (C14): C14 had a retention time of 50.8~51.3 min on HPLC. It

showed $[M-H]^-$ at m/z 267 in negative ion mass spectrum, and $[M+H]^+$ at m/z 269, $[M+Na]^+$ at m/z 291, $[2M+Na]^+$ at m/z 559 in positive ion mass spectrum. So, its molecular weight was inferred to be 268 Da. Its UV spectrum exhibited maximum absorption at 250, 288 nm and shoulder peak at 300 nm with weak band I (300 nm) and strong band II (250 nm), which suggested that it was an isoflavone. The negative MS^2 spectrum of m/z 267 showed fragment ions at m/z 252 and m/z 224; sequential loss of 15 Da and 28 Da from the precursor ion indicated the presence of a methyl and a carbonyl in the molecule. The positive MS^2 spectrum of m/z 291 showed a fragment ion at m/z 273. The fragment ion at m/z 273, loss of 18 Da from ion m/z 291, suggested the presence of a hydroxyl group. In the chemical database of Astragali Radix, only formononetin had a molecular weight of 268 Da. Based on these data, C14 was identified as formononetin tentatively.

Compound 15 (C15): C15 had a retention time of 51.9~52.3 min on HPLC. It showed $[M-H]^-$ at m/z 299 in negative ion mass spectrum, and $[M+H]^+$ at m/z 301, $[M+Na]^+$ at m/z 323, $[2M+Na]^+$ at m/z 623 in positive ion mass spectrum. So, its molecular weight was inferred to be 300 Da. Its UV spectrum exhibited maximum absorption at 208, 280 nm, shoulder peak at 225 nm and minimum absorption at 250 nm, which suggested that it was an isoflavan or a pterocarpan. The negative MS^2 spectrum of m/z 299 showed fragment ions at m/z 284, 269, 241; sequential loss of 15 Da, 15 Da and 28 Da from m/z 299 indicated the presence of two methyl groups and a C-O fragment in the molecule. In the chemical database of Astragali Radix, three compounds, i.e., (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan,

(6aR,11aR)-3,9-dimethoxy-10-hydroxypterocarpan and 3,4',5-trihydroxy-7-methoxyflavone, have the molecular weight of 300 Da, but only (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan was a pterocarpan isolated from *Astragalus membranaceus* var. *mongholicus*. Based on these data, C15 was identified as (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan.

Compound 16 (C16): C16 had a retention time of 52.4~52.7 min on HPLC. It showed $[M-H]^-$ at m/z 301 in negative ion mass spectrum, indicating that its molecular weight was 302 Da. Its UV spectrum exhibited maximum absorption at 204, 280 nm, shoulder peak at 225 nm and minimum absorption at 250 nm, suggesting that it was an isoflavan or a pterocarpan. The negative MS^2 spectrum of m/z 301 gave fragment ions at m/z 286, 153, 147, 135, 121, 109. The fragment ion at m/z 286, loss of 15 Da from the precursor ion indicated that there was a methyl in the molecule. The fragment ions at m/z 147 and m/z 153 were a pair of complementary ions, m/z 153 was from B ring of isoflavan, m/z 147 was from A ring and C ring. The characteristic A ring ions at m/z 121, 109 were generated by Retro Diels-Alder fragmentation in C ring. The fragmentation ion at m/z 135 was also from A ring and C ring by loss of B ring and C_3 from the quasi-molecular ion at m/z 301. These data indicated that C16 was an isoflavan, and there was only one hydroxyl substituent on A ring. In the chemical database of Astragali Radix, three compounds, i.e., 7,2'-dihydroxy-3',4'-dimethoxyisoflavan, quercetin and (3R)-8,2'-dihydroxy-7,4'-dimethoxyisoflavan, have the molecular weight of 302 Da, but only 7,2'-dihydroxy-3',4'-dimethoxyisoflavan was an isoflavan with one

substituent on A ring isolated from *Astragalus membranaceus* var. *mongholicus*.

Based on these data, C16 was identified as 7,2'-dihydroxy-3',4'-dimethoxyisoflavan tentatively.

Compound 19 (C19): C19 had a retention time of 51.4~51.7 min on HPLC. It showed $[M-H]^-$ at m/z 331 in negative ion mass spectrum, indicating that its molecular weight was 332 Da. Its UV spectrum exhibited maximum absorption at 204, 280 nm, shoulder peak at 225 nm and minimum absorption at 250 nm, suggesting that it was an isoflavan or a pterocarpan. The molecular weight of C19 was only 30 Da higher than that of 7,2'-dihydroxy-3',4'-dimethoxyisoflavan (C16), suggesting that it might be a methoxyl derivative of C16. The negative MS^2 spectrum of m/z 331 gave fragment ions at m/z 316, 301, 299, 313, 295, 209, 194, 147, 135, 109. The fragment ions at m/z 316, 301, sequential loss of 15 Da, 15 Da from m/z 331 indicated the presence of two methyl groups. The fragment ions at m/z 313, 295, sequential loss of 18 Da, 18 Da from m/z 331 indicated the presence of two hydroxy groups. The fragment ion m/z 299, loss of 32 Da from m/z 331 indicated that there was a 2' or 6' methoxyl in the molecule. The characteristic B ring ions at m/z 209, 194 were generated by Retro Diels-Alder fragmentation in C ring. The fragmentation ion m/z 147 was from A ring and C ring. The fragmentation ion at m/z 135 was also from A ring and C ring by loss of B ring and C_3 from the quasi-molecular ion at m/z 301. The fragment ion at m/z 109 was from A ring, indicating that there was only one hydroxy in A ring. These data indicated that C19 was an isoflavan with at least two methyl, two hydroxy and one 2' or 6' methoxyl in the molecule. In the chemical database of

Astragali Radix, no compound has a molecular weight of 332 Da. Based on these data and compared with the structure of 7,2'-dihydroxy-3',4'-dimethoxyisoflavan (C16), C19 was tentatively identified as a 6' methoxyl substitution derivative of C16, i.e., 7,2'-dihydroxy-3',4',6'-trimethoxyisoflavan, and this compound was found in Astragali Radix for the first time.

Compound 8 (C8): C8 had a retention time of 30.9~31.7 min on HPLC. It showed $[M-H]^-$ at m/z 445, $[M+HCOOH-H]^-$ at m/z 491, $[M+CH_3COOH-H]^-$ at m/z 505 and aglycon ion at m/z 283 in negative ion mass spectrum. It showed $[M+H]^+$ at m/z 447, $[M+Na]^+$ at m/z 469, $[M+K]^+$ at m/z 485, $[2M+Na]^+$ at m/z 915 in positive ion mass spectrum. So, its molecular weight was inferred to be 446 Da. Its UV spectrum exhibited maximum absorption at 200, 220, 250, 258 nm and shoulder peak at 286 nm with weak band I (300~400 nm) and strong band II (250 nm), suggesting that it was an isoflavone. The positive MS^2 spectrum of m/z 447 gave a fragment ion at m/z 285; loss of 162 Da indicated that it was a glucoside. The positive MS^3 spectrum of m/z 285 showed fragment ions at m/z 270, 267, 257, 253, 225, 137. The fragment ions at m/z 270, 253 and 225, sequential loss of 15 Da, 17 Da, 28 Da from aglycon ion m/z 285, suggested the presence of a methyl, a hydroxyl and a carbonyl group. The fragment ion at m/z 137 was derived from A ring of isoflavone. These data were identical with those of calycosin-7-*O*- β -D-glucoside reported in the literature (Xiao et al., 2004). In addition, the HPLC retention time, UV spectrum, MS^n data of C8 were identical with those of standard compound of calycosin-7-*O*- β -D-glucoside. So, C8 was identified as calycosin-7-*O*- β -D-glucoside unequivocally.

Compound 9 (C9): C9 had a retention time of 38.6~38.9 min on HPLC. It showed $[M-H]^-$ at m/z 429, $[M+HCOOH-H]^-$ at m/z 475, $[M+CH_3COOH-H]^-$ at m/z 489, $[M+Cl]^-$ at m/z 465, $[2M-H]^-$ at m/z 859 and aglycon ion at m/z 267 in negative ion mass spectrum. It showed $[M+H]^+$ at m/z 431, $[M+Na]^+$ at m/z 453 and $[M+K]^+$ at m/z 469 in positive ion mass spectrum. So, its molecular weight was inferred to be 430 Da. Its UV spectrum exhibited maximum absorption at 254 nm and shoulder peak at 300 nm with weak band I (300~400 nm) and strong band II (254 nm), suggesting that it was an isoflavone. The negative MS^2 spectrum of m/z 489 showed ions at m/z 429, 267; loss of 162 Da indicated that it was a glucoside. The negative MS^3 spectrum of m/z 267 showed fragment ions at m/z 252, 223; sequential loss of 15 Da, 29 Da from aglycon ion m/z 267 suggested the presence of a methyl and a carbonyl group. Only formononetin-7-*O*- β -D-glucoside had a molecular weight of 430 Da in the chemical database of Astragali Radix. Based on these data, C9 was identified as formononetin-7-*O*- β -D-glucoside.

Compound 11 (C11): C11 had a retention time of 40.2~40.9 min on HPLC. It showed $[M-H]^-$ at m/z 461, $[M+HCOOH-H]^-$ at m/z 507, $[M+CH_3COOH-H]^-$ at m/z 521 and $[2M-H]^-$ at m/z 923 in negative ion mass spectrum. It showed $[M+H]^+$ at m/z 463, $[M+NH_4]^+$ at m/z 480, $[M+Na]^+$ at m/z 485 and $[M+K]^+$ at m/z 501 in positive ion mass spectrum. So, its molecular weight was inferred to be 462 Da. Its UV spectrum exhibited maximum absorption at 206, 284 nm, shoulder peak at 230 nm and minimum absorption at 250 nm, suggesting that it was an isoflavan or a pterocarpan. The negative MS^2 spectrum of m/z 461 showed fragment ions at m/z 299,

284; sequential loss of 162 Da and 15 Da indicated the presence of a glucosyl and a methyl group. The negative MS³ spectrum of m/z 299 showed fragment ions at m/z 284, 269; sequential loss of 15 Da, 15 Da indicated the presence of two methyl groups in the molecule. In the chemical database of Astragali Radix, (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside, rhamnocitrin-3-*O*- β -D-glucoside and pratensein-7-*O*- β -D-glucoside have the molecular weight of 462 Da, but only (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside was a pterocarpan isolated from *Astragalus membranaceus* var. *mongholicus*. Based on these data, C11 was identified as (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside.

Compound 12 (C12): C12 had a retention time of 40.8~41.8 min on HPLC. It showed $[M-H]^-$ at m/z 463 in negative ion mass spectrum, and $[M+NH_4]^+$ at m/z 482, $[M+Na]^+$ at m/z 487 in positive ion mass spectrum. This indicated that its molecular weight was 464 Da. Its UV spectrum exhibited maximum absorption at 204, 280 nm, shoulder peak at 226 nm and minimum absorption at 250 nm, suggesting that it was an isoflavan or a pterocarpan. The negative MS² spectrum of m/z 463 showed fragment ions at m/z 301, 286, 271; sequential loss of 162 Da, 15 Da and 15 Da indicated the presence of a glucosyl and two methyl groups. The negative MS³ spectrum of m/z 301 gave fragment ions at m/z 286, 254, 179, 164, 153, 147, 135, 121, 109. The fragment ions at m/z 121 and m/z 179 were a pair of complementary ions generated by Retro Diels-Alder fragmentation in C ring. The fragment ions at m/z 147

and m/z 153 were a pair of complementary ions, m/z 153 was from B ring, m/z 147 was from A ring and C ring. The ion at m/z 109 was from A ring. The fragmentation ion at m/z 135 was also from A ring and C ring by loss of B ring and C_3 from the quasi-molecular ion at m/z 301. The fragmentation ion at m/z 254, loss of 32 Da from m/z 286, indicated the presence of a 2' or 6' methoxyl in the molecule. The positive MS^2 spectrum of m/z 482 showed fragmentation ions at m/z 465, 303. The positive MS^3 spectrum of m/z 303 gave fragmentation ions at m/z 193, 181, 167, 149, 123. These positive ion mass data also indicated that C12 was an isoflavan glucoside. In the chemical database of Astragali Radix, 7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside, quercetin-3-*O*- β -D-glucoside and 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside have a molecular weight of 464 Da, but only 7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside was an isoflavan with a 2' methoxyl. Based on these data, C12 was identified as 7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside tentatively.

Compound 10 (C10): C10 had a retention time of 39.0~39.4 min on HPLC. It showed $[M-H]^-$ at m/z 593 in negative ion mass spectrum. It showed $[M+NH_4]^+$ at m/z 612 and $[M+Na]^+$ at m/z 617 in positive ion mass spectrum. So, its molecular weight was confirmed to be 594 Da. Its UV spectrum exhibited maximum absorption at 207, 280 nm, shoulder peak at 230 nm and minimum absorption at 250 nm, suggesting that it was an isoflavan or a pterocarpan. The negative MS^2 spectrum of

m/z 593 showed fragment ions at m/z 461, 299, 284, 269, 241; sequential losses of 132 Da, 162 Da, 15 Da, 15 Da and 28 Da indicated the presence of a pentosyl, a glucosyl, two methyls and a C-O fragment. The positive MS^2 spectrum of m/z 617 showed fragment ions at m/z 485, 323, 317, indicating that the glucosyl was directly attached to the aglycon, and the pentosyl was linked to the glucosyl group. There were no compounds whose molecular weight was 594 Da in the chemical database of Astragali Radix. By searching the constituents of Astragalus plants in the Combined Chemical Dictionary on CD-ROM version 8.1, we found that the common glucosyl-pentosyl residue in Astragalus plants was sambubiose. Based on these data, C10 was identified as (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-sambubioside tentatively, and this compound was found in Astragali Radix for the first time.

Compound 6 (C6): C6 had a retention time of 24.5~24.9 min on HPLC. It showed $[M-H-CO_2]^-$ at m/z 503 and aglycon ion at m/z 299 in negative ion mass spectrum. It showed $[M+H]^+$ at m/z 549, $[M+NH_4]^+$ at m/z 566, $[M+Na]^+$ at m/z 571, $[M+K]^+$ at m/z 587 in positive ion mass spectrum. So, its molecular weight was inferred to be 548 Da. Its UV spectrum exhibited maximum absorption at 280 nm and minimum absorption at 250 nm, suggesting that it was an isoflavan or a pterocarpan. The positive MS^2 spectrum of m/z 571 showed fragment ions at m/z 527, 485 and m/z 323; loss of 44 Da, 86 Da from the ion at m/z 571 and loss of 162 Da from the ion at m/z 485 indicated the presence of a malonyl and a glucosyl in the molecule. The negative MS^2 spectrum of m/z 503 gave fragmentation ions at m/z 459, 299, 284. The negative

MS³ spectrum of m/z 299 gave fragment ions at m/z 284, 269. These data indicated that the aglycon of C6 had a molecular mass of 300 Da and it contained two methyl groups. In the chemical database of Astragali Radix, only (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside-6''-*O*-malonate had a molecular weight of 548. Based on these data, C6 was identified as (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside-6''-*O*-malonate tentatively.

Compound 7 (C7): C7 had a retention time of 26.2~26.9 min on HPLC. It showed $[M-H-CO_2]^-$ at m/z 505 in negative ion mass spectrum, $[M+NH_4]^+$ at m/z 568, $[M+Na]^+$ at m/z 573 in positive ion mass spectrum, indicating its molecular weight was 550 Da. Its UV spectrum exhibited maximum absorption at 204, 280 nm, shoulder peak at 225 nm and minimum absorption at 250 nm, suggesting that it was an isoflavan or a pterocarpan. The negative MS² spectrum of m/z 505 gave fragmentation ions at m/z 463, 445, 427, 399, 301, 286, 179. These data indicated that C7 was a glycoside, and its aglycon had a molecular mass of 302 Da and at least contained one methyl. The positive MS² spectrum of m/z 573 showed fragment ions at m/z 529, 487; loss of 44 Da, 86 Da from the precursor ion indicated the presence of a malonyl in the molecule. In the chemical database of Astragali Radix, only 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside-6''-*O*-malonate had a molecular weight of 550. Based on these data, C7 was identified as 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside-6''-*O*-malonate tentatively.

Identification of the metabolites of absorbable compounds

Compound 1 (C1): C1 had a retention time of 19.2~20.2 min on HPLC. It showed $[M-H]^-$ at m/z 459 in negative ion mass spectrum, showed $[M+H]^+$ at m/z 461, $[M+Na]^+$ at m/z 483, $[M+K]^+$ at m/z 499 in positive ion mass spectrum. So, its molecular weight was inferred to be 460 Da. Its UV spectrum exhibited maximum absorption at 198, 250 nm and shoulder peak at 216, 288, 305 nm with weak band I (305 nm) and strong band II (250 nm), suggesting that it was an isoflavone. The negative MS^2 spectrum of m/z 459 gave fragment ions at m/z 441, 415, 283, 268, 175, indicating that C1 was a glucuronide and its aglycon had a molecular mass of 284 Da and there was a methyl in the aglycon. The negative MS^3 spectrum of m/z 175 gave fragment ions at m/z 117, 113, confirmed that it was a glucuronosyl group (Chen et al., 1998). Based on these data, C1 was identified as calycosin-7-*O*- β -D-glucuronide.

Compound 2 (C2): C2 had a retention time of 21.3~22.1 min on HPLC. It showed $[M-H]^-$ at m/z 475 in negative ion mass spectrum, showed $[M+H]^+$ at m/z 477, $[M+Na]^+$ at m/z 499 in positive ion mass spectrum. So, its molecular weight was inferred to be 476 Da. Its UV spectrum exhibited maximum absorption at 206, 280 nm, shoulder peak at 225 nm and minimum absorption at 262 nm, suggesting that it was an isoflavan or a pterocarpan. The negative MS^2 spectrum of m/z 475 showed fragment ions at m/z 457, 299, 284, 269, 175, 157, which indicated that C2 was a glucuronide and its aglycon had a molecular mass of 300 Da and there were two methyl groups in the aglycon. The negative MS^3 spectrum of m/z 175 gave fragment ions at m/z 117, 113, confirming that it was a glucuronosyl group (Chen et al., 1998).

Based on these data, C2 was identified as (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucuronide.

Compound 3 (C3) and compound 5 (C5): C3 had a retention time of 22.6~22.9 min on HPLC. C5 had a retention time of 23.6~24.0 min on HPLC. They showed $[M-H]^-$ at m/z 477 in negative ion mass spectrum and $[M+Na]^+$ at m/z 501 in positive ion mass spectrum. So, their molecular weights were inferred to be 478 Da. Their UV spectra exhibited maximum absorption at 204, 280 nm, shoulder peak at 226 nm and minimum absorption at 250 nm, suggesting that they were isoflavans or pterocarpan. The negative MS^2 spectra of m/z 477 (from C3 and C5) showed fragment ions at m/z 459, 301, 286, 271, 175, 157, 147, 135, 121, 113, 109, indicating that C3 and C5 were isoflavan glucuronides and their aglycons had a molecular mass of 302 Da with two methyl groups in the aglycons. The negative MS^3 spectrum of m/z 175 gave fragment ions at m/z 117, 113, which confirmed that it was a glucuronosyl group (Chen et al., 1998). From figure 1, we can find that the peak area of C3 was smaller than that of C5. According to the literature (Chen et al., 2005), glucuronidation mainly occurred at the 7-hydroxy group. Besides, the ClogP of C5 was 0.356 and ClogP of C3 was 0.015, which indicated that C5 was more lipophilic than C3, so C5 should have a longer retention time than C3 on reverse phase HPLC. Thus C3 was identified as 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-2'-*O*- β -D-glucuronide and C5 was identified as 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucuronide tentatively.

Compound 4 (C4): C4 had a retention time of 23.0~23.3 min on HPLC. It showed $[M-H]^-$ at m/z 507 in negative ion mass spectrum and $[M+Na]^+$ at m/z 531 in positive

ion mass spectrum. So, its molecular weight was inferred to be 508 Da. Its UV spectrum exhibited maximum absorption at 203, 280 nm, shoulder peak at 226 nm and minimum absorption at 250 nm, suggesting that it was an isoflavan or a pterocarpan. The negative MS² spectrum of m/z 507 showed fragment ions at m/z 489, 463, 445, 401, 331, 316, 301, 175, 157, 113. The fragment ions at m/z 113, m/z 157, m/z 175 and m/z 331 indicated that C4 was a glucuronide and its aglycon had a molecular weight of 331 Da. The fragment ions at m/z 316, 301, sequential loss of 15 Da, 15 Da from m/z 331 indicated the presence of two methyls in the aglycon. The ions at m/z 489 [M-H-H₂O]⁻, 463 [M-H-CO₂]⁻, 445 [M-H-H₂O-CO₂]⁻ indicated the presence of a carboxyl in the molecule. The retention time of C4 (23.0~23.3 min) was between 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-2'-O-β-D-glucuronide (C3, 22.6~22.9 min) and 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-O-β-D-glucuronide (C5, 23.6~24.0 min), and the molecular weight of C4 (508 Da) was only 30 Da higher than those of C3, C5 (478 Da), these suggested that it might be a methoxyl derivative of C3 or C5 with a ClogP between 0.015 and 0.356. Besides, no loss of 32 Da was observed compared to 7,2'-dihydroxy-3',4',6'-trimethoxyisoflavan (C19), which indicated the absence of a 2' or 6' methoxyl in the molecule. Based on these data, 10 possible structures of C4 and their ClogP were shown in Supplemental data – Fig. s2. Among them, structure 1, 2 and 6 not only have a ClogP between 0.015 and 0.356, but also lack a 2' or 6' methoxyl. Moreover, methoxyl substitution at carbon-5 position of isoflavan was seldom. Thus the most possible structure of C4 was identified as 7,2'-dihydroxy-3',4',5'-trimethoxyisoflavan-7-O-β-D-glucuronide tentatively.

Compound 17 (C17): C17 had a retention time of 20.0~20.2 min on HPLC. It showed $[M-H]^-$ at m/z 443 in negative ion mass spectrum, which indicated that its molecular weight was 444 Da. Its UV spectrum exhibited maximum absorption at 208, 248 nm and shoulder peak at 284 nm. The band I (300~400 nm) was weak and the band II (250 nm) was strong, suggesting that it was an isoflavone. The negative MS^2 spectrum of m/z 443 gave fragment ions at m/z 267, 175, indicating that C17 was a glucuronide and its aglycon had a molecular mass of 268 Da. In the chemical database of Astragali Radix, only formononetin was found to have a molecular weight of 268 Da. Based on these data and our previous report (Yang et al., 2006), C17 was identified as formononetin-7-*O*- β -D-glucuronide tentatively.

Compound 18 (C18): C18 had a retention time of 21.1~21.6 min on HPLC. It showed $[M-H]^-$ at m/z 363 in negative ion mass spectrum, indicating that its molecular weight was 364 Da. Its UV spectrum exhibited maximum absorption at 250 nm and shoulder peak at 305 nm with weak band I (305 nm) and strong band II (250 nm), suggesting that it was an isoflavone. The negative MS^2 spectrum of m/z 363 gave fragment ions at m/z 283 $[M-H-SO_3H]^-$, 268 $[M-H-SO_3H-CH_3]^-$, 135, which indicated that C18 was a sulphate and its aglycon had a molecular mass of 284 Da with a methyl in the aglycon. The ion at m/z 135 derived from A ring of isoflavones was formed from retro Diels-Alder fragmentation in C ring (Xiao et al., 2004). Furthermore, the isotopic abundance ratio of m/z 365 to m/z 363 was 8.5% (137208 to 1611981) in negative ion mass spectrum, which suggested that there was one sulfur atom in the molecule. Based on these data, C18 was identified as calycosin sulphate

tentatively.

Compound 20 (C20): C20 had a retention time of 17.3~17.8 min on HPLC. It showed $[M-H]^-$ at m/z 429 in negative ion mass spectrum, indicating that its molecular weight was 430 Da. The negative MS^2 spectrum of m/z 429 gave fragment ions at m/z 253, 175, 157, which indicated that C20 was a glucuronide and its aglycon had a molecular mass of 254 Da. Based on these data, C20 was tentatively identified as a demethylating metabolite of formononetin, i.e., daidzein-7-*O*- β -D-glucuronide (Lania-Pietrzak et al., 2005).

Compound 21 (C21): C21 had a retention time of 23.1~23.5 min on HPLC. It showed $[M-H]^-$ at m/z 639 in negative ion mass spectrum and $[M+NH_4]^+$ at m/z 658, $[M+Na]^+$ at m/z 663 in positive ion mass spectrum, which indicated that its molecular weight was 640 Da. The positive MS^2 spectrum of m/z 658 showed fragment ions at m/z 641, 479, 465, 303. The positive MS^3 spectrum of m/z 303 gave fragment ions at m/z 193, 181, 167, 149, 123; these data were identical with those of 7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7-*O*- β -D-glucoside (C12). The positive MS^2 spectrum of m/z 663 showed a fragment ion at m/z 487. The positive MS^3 spectrum of m/z 487 gave fragment ions at m/z 472, 325, 302, 185. The negative MS^2 spectrum of m/z 639 gave fragmentation ions at m/z 621, 607, 463, 301. The fragmentation ion at m/z 607, loss of 32 Da from m/z 639, indicated the presence of a 2' or 6' methoxyl in the molecule. These data indicated that C21 was a glucuronide of isoflavan glucoside, and its aglycon had a molecular mass of 302 Da. Based on these data, C21 was tentatively identified as

7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7-*O*- β -D-glucosyl-3'-*O*- β -D-glucuronide.

Discussion

In present study, we reported the absorption and metabolism of Astragali Radix decoction for the first time. Four complementary methods, i.e., computational chemistry prediction method, Caco-2 cell monolayer model experiment, improved rat everted gut sac experiment and healthy human volunteer experiment were used. The results of four methods were compared in Table 1.

As shown in Table 1, it was found that in four methods, the main absorbable constituents of Astragali Radix decoction were flavonoids. Calycosin (C13), formononetin (C14), (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan (C15), 7,2'-dihydroxy-3',4'-dimethoxyisoflavan (C16), calycosin-7-*O*- β -D-glucoside (C8), formononetin-7-*O*- β -D-glucoside (C9) and other six compounds could be detected and identified as absorbable constituents. On the other hand, calycosin-7-*O*- β -D-glucuronide (C1), (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucuronide (C2), 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucuronide (C5), formononetin-7-*O*- β -D-glucuronide (C17), calycosin sulphate (C18) and other four compounds were detected as metabolites of the constituents of Astragali Radix decoction.

Calycosin (C13) and formononetin (C14) were proved absorbable by four methods. (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan (C15) and 7,2'-dihydroxy-3',4'-dimethoxyisoflavan (C16) were proved absorbable by three methods; the existence of

(6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucuronide (C2), 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucuronide (C5) also implied that (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan (C15) and 7,2'-dihydroxy-3',4'-dimethoxyisoflavan (C16) were absorbable compounds. Formononetin-7-*O*- β -D-glucoside (C9) and 9,10-dimethoxypterocarpane-3-*O*- β -D-glucoside (C11) were proved absorbable by two methods. The existence of metabolites C1, C2, C5, C17, C18 was proved by two or three methods. No saponins were predicted to be oral absorbable compounds in *in silico* experiment, because the saponins in Astragali Radix always existed in the form of saponin glycosides with large molecular weight and high molecular polarity. In addition, no saponins of Astragali Radix were detected in *in vitro* and *in vivo* experiments. The reason might be: (i) the content of saponins was low in the decoction, (ii) the absorption of saponins was poor. For example, the absolute bioavailability of astragaloside IV in rat was only 2.2% (Gu et al., 2004), (iii) ion suppression phenomenon might exist, and the ionization of saponins was suppressed.

In *in vivo* human experiment, six flavonoid glucuronides (C1, C2, C5, C17, C20, C21), one isoflavone sulphate (C18) and two isoflavone aglycons (C13, C14) were found and identified in the drug-containing urine. The result indicated that after oral administration of Astragali Radix decoction, the major metabolites in human urine were flavonoid glucuronides. Three most abundant peaks were (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucuronide (C2), 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucuronide (C5) and calycosin

sulphate (C18), which suggested that their aglycons might be easily absorbed. The existence of

7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7-*O*- β -D-glucosyl-3'-*O*- β -D-glucuronide (C21) implied that isoflavan monoglycoside in Astragali Radix decoction also could be absorbed though the amount was small.

In *in vitro* Caco-2 cell monolayer model, five flavonoid aglycons (C13, C14, C15, C16, C19) and one isoflavone sulphate (C18) were detected and identified in the basolateral side solution. The result suggested that the main absorbable constituents of Astragali Radix decoction in this model were flavonoid aglycons. Two most abundant peaks were calycosin sulphate (C18) and calycosin (C13), suggesting that calycosin was easily absorbed and metabolized by the cell monolayer. That no flavonoid glycosides and only one metabolite were detected in the basolateral side solution implied that the absorption of glycoside in Caco-2 model and the metabolic ability of Caco-2 cell monolayer were poor.

In *in vitro* improved rat everted gut sac experiment, 18 compounds were found and identified in the serosal side solution. All were flavonoids, including four flavonoid aglycons, seven flavonoid glycosides, six flavonoid glucuronides and one flavonoid sulphate. The result showed that: **(i)** the main absorbable constituents of Astragali Radix decoction in this experiment were flavonoids; **(ii)** both flavonoid aglycon and flavonoid glycoside could be absorbed by rat intestine; **(iii)** flavonoids could be metabolized by intestine during absorption process, the major metabolites were glucuronides and the minor were sulphates; **(iv)** although it had been reported that

flavone, flavonol, flavanones (such as apigenin, luteolin, quercetin, kaempferol, hesperetin) including their glucosides (Hu et al., 2003; Spencer et al., 1999) and isoflavones (such as genistein, daidzein, formononetin) could be absorbed and glucuronidated by the small intestine (Chen, et al., 2005; Liu and Hu, 2002), we found that pterocarpan (C15), isoflavan (C16), calycosin (C13) also could be glucuronidated by the small intestine for the first time; (v) no phase I metabolites were detected, which suggested that phase I metabolism of flavonoids during absorption was poor; (vi) the metabolites (C1, C2, C3, C4, C5, C17, C18) were also detected in mucosal solution, suggesting that glucuronides (C1, C2, C3, C4, C5, C17) and sulphates (C18) could be excreted to the mucosal side; (vii) two most abundant peaks were calycosin-7-*O*- β -D-glucuronide (C1) and 7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7-*O*- β -D-glucuronide (C5), which implied that their aglycons were easily absorbed by rat intestine.

In the *in silico* computational chemistry prediction method, 26 compounds were regarded as oral available compounds, including 12 flavonoids, five phenolic acids, five nitrogen-containing compounds, three lignanoids and one coumarin. The flavonoids almost accounted for 50 % of the oral absorbable compounds.

In *in vivo* human experiment, two isoflavone aglycons (C13, C14) agreed with the *in silico* prediction. In Caco-2 model, four flavonoid aglycons (C13, C14, C15, C16) coincided with the *in silico* prediction. In everted gut sac experiment, six original compounds (C9, C11, C13, C14, C15, C16) coincided with *in silico* prediction. Therefore, *in silico* computational chemistry prediction method could be used to

pre-screen oral available compounds, and it was a time and money saving method. The disadvantage was that it could not predict the compounds absorbed by active transport, and it did not consider the concentrations of the compounds.

In *in vivo* human experiment, seven compounds (C13, C14, C18, C1, C17, C2, C5) were identical with those identified in everted gut sac experiment. In Caco-2 model, five compounds (C13, C14, C15, C16, C18) were identical with those identified in everted gut sac experiment. This suggested that everted gut sac model was a good *in vitro* model. This organ model was more similar to *in vivo* situation, and its phase II metabolic ability was stronger than Caco-2 cell monolayer. In addition, the experimental time was short and the cost was low. The disadvantage was that it was a rat model, and species difference might exist.

Caco-2 cell monolayer model was the most popular cellular model in studies on passage and transport. The cell line was from human, so it could be used to predict human intestinal absorption of drugs, but the preparation time of this experiment was long, and the cost was high. The advantage of human volunteer experiment was that it was *in vivo* experiment. The drawback was that it needs a great amount of compound or crude drug, and it was not a universal method, e.g., it could not be used to study the absorption and metabolism of toxic crude drugs. In addition, it was an indirect drug absorption research method, and we had to deduce the absorbed compounds from their metabolites. Therefore, we used these four methods in present study simultaneously.

Our previous *in vitro* pharmacological study proved that calycosin,

calycosin-7-*O*- β -D-glucoside, formononetin and (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside were able to increase the fluidity of brain cell membrane in ischemia-reperfusing rats to resume or close to normal control level (Li et al., 2001). It has been proved that formononetin, (6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-*O*- β -D-glucoside, formononetin-7-*O*- β -D-glucoside, calycosin-7-*O*- β -D-glucoside and calycosin have neuroprotective and antioxidant effects (Yu et al., 2005). Formononetin also showed an inhibitory effect on mouse brain monoamine oxidase (Hwang et al., 2005). Calycosin could protect endothelial cells from hypoxia-induced barrier impairment (Fan et al., 2003). The aglycon of daidzein-7-*O*- β -D-glucuronide, i.e., daidzein, and formononetin were phytoestrogens, which had many well-known pharmacological activities. Furthermore, it has been reported that daidzein and formononetin have immunological enhancement actions (Zhang and Han, 1994). These bioactivities were relevant to the curative effect of Astragali Radix decoction. Thus, we concluded that these identified absorbable compounds were an important component part of the active substances of Astragali Radix decoction. There might be other bioactive substances of Astragali Radix, which needs further investigation.

In summary, our study had demonstrated that the flavonoids in Astragali Radix decoction, including isoflavones, pterocarpan and isoflavans, could be absorbed and metabolized by intestine. Totally 21 compounds were identified, including 5 flavonoid aglycons, 7 flavonoid glycosides, 8 flavonoid glucuronides and 1 isoflavone sulphate.

They were mainly calycosin, formononetin,

(6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan,
7,2'-dihydroxy-3',4'-dimethoxyisoflavan and their glycosides and phase II metabolites.
No phase I metabolites were detected in the study and the main metabolites were glucuronides. In addition to isoflavones, we found that pterocarpan and isoflavans also could be metabolized by the intestine during absorption for the first time. The absorbable compounds identified in present study, namely the flavonoids, had many bioactivities related to the curative effect of Astragali Radix decoction. So, they could be regarded as an important component part of the active substances of Astragali Radix decoction.

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Footnotes

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Legends for figures

Figure 1. *Representative HPLC-MS base peak chromatograms of the samples at 60 min in improved rat everted gut sac experiment detected in negative ion mode. Blank serosal solution (A), Astragali Radix serosal solution (B) and Astragali Radix mucosal solution (C)*

C1~C18 denote compound 1 to compound 18.

Figure 2. *Representative HPLC-MS base peak chromatograms of the samples at 180 min in Caco-2 experiment detected in negative ion mode. Astragali Radix apical solution (A), Blank basolateral solution (B) and Astragali Radix basolateral solution (C)*

C13, C14, C15, C16, C18, C19 denote compound 13, 14, 15, 16, 18, 19 respectively.

Figure 3. *Representative extracted ion chromatograms at m/z 363, 475, 477, 443, 459, 429, 639, 283, 267 of blank urine (A) and drug-containing urine (B) of a male volunteer orally administrated Astragali Radix decoction detected in negative ion mode.*

C1, C2, C5, C17, C18, C20, C21 denote compound 1, 2, 5, 17, 18, 20, 21 respectively.

Figure 4. *Structures of 21 compounds identified in the in vitro and in vivo absorption and metabolism experiments of Astragali Radix decoction*

TABLE 1

Comparison of absorbable compounds and their metabolites of Astragali Radix decoction identified in four methods: in silico prediction, improved rat everted gut sac model, Caco-2 cell monolayer model and human volunteer experiment

Structure type	Compound Name	<i>In silico</i> prediction	everted gut sac	Caco-2 model	Human urine
flavonoid	formononetin (C14)	√	√	√	√
aglycon	calycosin (C13)	√	√	√	√
	(6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan (C15)	√	√	√	
	7,2'-dihydroxy-3',4'-dimethoxyisoflavan (C16)	√	√	√	
	7,2'-dihydroxy-3',4',6'-trimethoxyisoflavan (C19) ^a			√	
	(3R)-2'-hydroxy-7,3',4'-trimethoxy isoflavan	√			
	(6aR, 11aR)-3,9,10-trimethoxypterocarpan	√			
	quercetin	√			
	isorhamnetin	√			
	rhamnocitrin	√			
	kaempferol	√			
flavonoid	(6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpane-3-O-β-D-g	√	√		
glycoside	lucoside (C11)				
	formononetin-7-O-β-D-glucoside (C9)	√	√		
	(6aR,11aR)-3-hydroxy-9,10-dimethoxypterocarpan-3-O-β-D-gl		√		
	ucoside-6''-O-malonate(C6)				

DMD #8300

	7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7- <i>O</i> -β-D-glucoside-6''	√		
	- <i>O</i> -malonate (C7)			
	calycosin-7- <i>O</i> -β-D-glucoside (C8)	√		
	(6a <i>R</i> ,11a <i>R</i>)-3-hydroxy-9,10-dimethoxypterocarpan-3- <i>O</i> -β-D-sa	√		
	mbubioside (C10) ^a			
	7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7- <i>O</i> -β-D-glucoside	√		
	(C12)			
flavonoid	calycosin sulphate (C18)	√	√	√
metabolite ^b	calycosin-7- <i>O</i> -β-D-glucuronide (C1)	√		√
	formononetin-7- <i>O</i> -β-D-glucuronide (C17)	√		√
	(6a <i>R</i> ,11a <i>R</i>)-3-hydroxy-9,10-dimethoxypterocarpan-3- <i>O</i> -β-D-	√		√
	glucuronide (C2)			
	7,2'-dihydroxy-3',4'-dimethoxyisoflavan-2'- <i>O</i> -β-D-glucuronide	√		
	(C3)			
	7,2'-dihydroxy-3',4',5'-trimethoxyisoflavan-7- <i>O</i> -β-D-glucuroni	√		
	de (C4)			
	7,2'-dihydroxy-3',4'-dimethoxyisoflavan-7- <i>O</i> -β-D-glucuronide	√		√
	(C5)			
	7,3'-dihydroxy-2',4'-dimethoxyisoflavan-7- <i>O</i> -β-D-glucosyl-3'-			√
	<i>O</i> -β-D-glucuronide (C21)			
	daidzein-7- <i>O</i> -β-D-glucuronide (C20)			√
Nitrogen	γ-aminobutyric acid	√		

DMD #8300

containing	3-hydroxy-2-methylpyridine	√
compound	nicotinic acid	√
	HDTIC ^c -1	√
	HDTIC ^c -2	√
lignanoid	bifendate	√
	(+)-lariciresinol	√
	(-)-syringaresinol	√
phenolic	vanillic acid	√
acid	isoferulic acid	√
	caffeic acid	√
	ferulic acid	√
	<i>p</i> -hydroxycinnamic acid	√
coumarin	coumarin	√

^a The compound was detected in Astragali Radix for the first time, and its oral availability was not predicted.

^b Drug-like properties of flavonoid metabolites were not calculated, and their oral availabilities were not predicted.

^c HDTIC: 4-Hydroxy-5-hydroxymethyl-[1,3]dioxolan-2,6'-spirane-5',6',7',8'-tetrahydro-indolizine-3'-carbaldehyde

Fig. 1

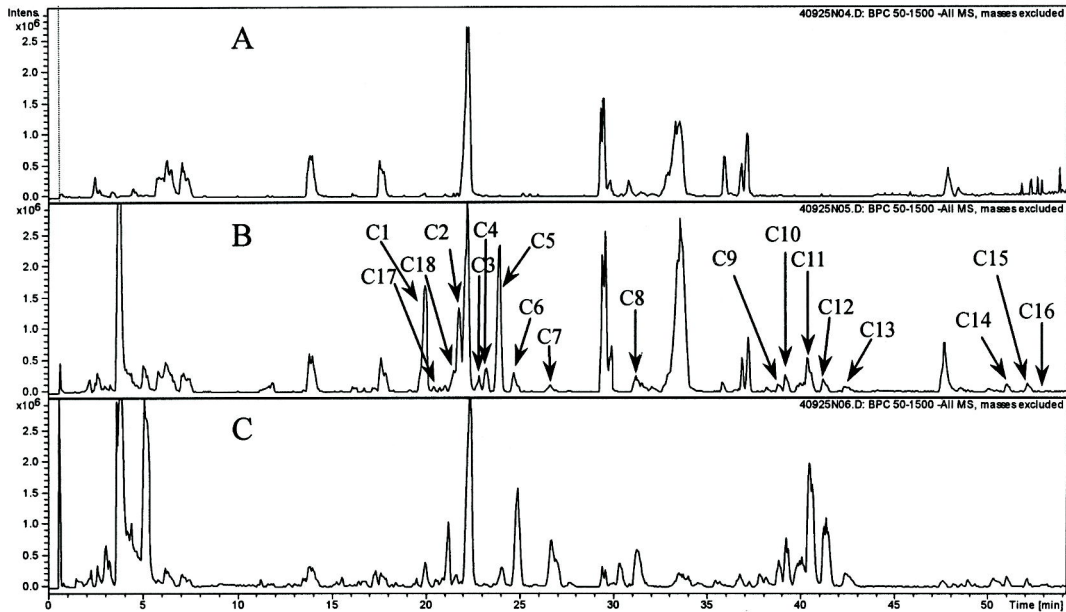


Fig. 2

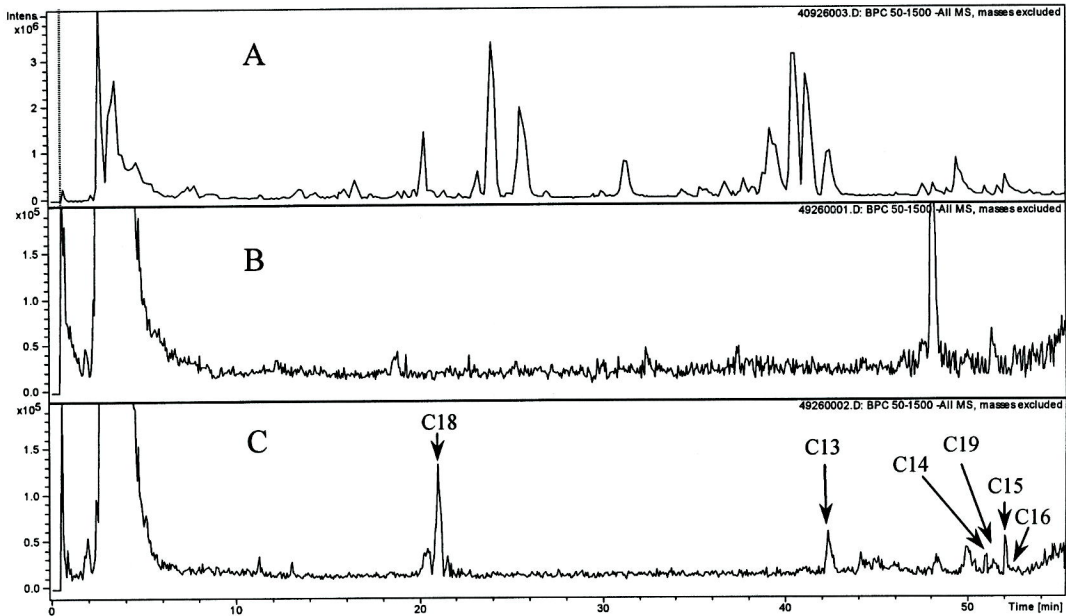
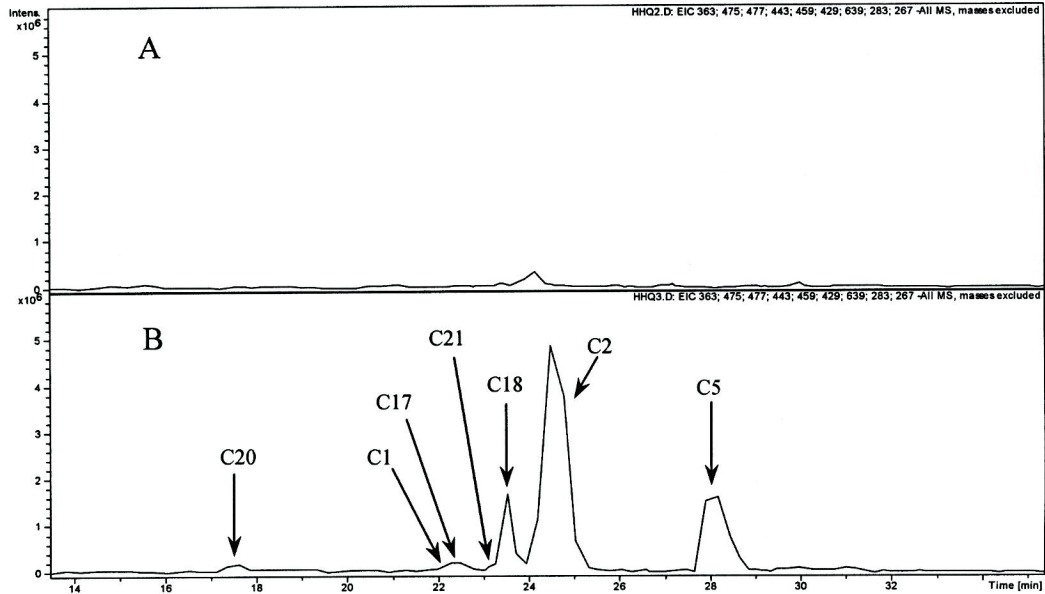
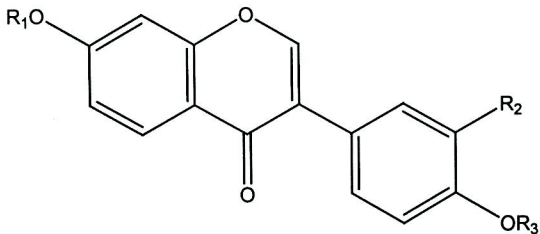
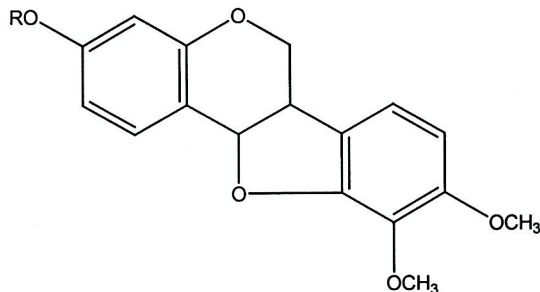


Fig. 3

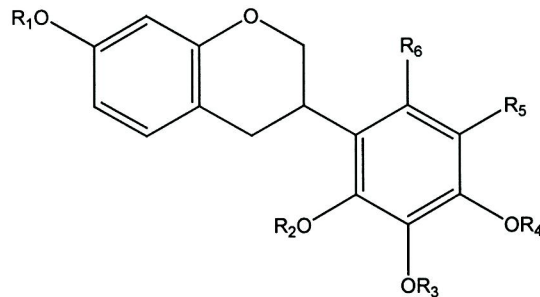




formononetin(C14): $R_1=R_2=H$, $R_3=CH_3$
 formononetin-7-*O*-beta-D-glucoside(C9): $R_1=\text{glucosyl}$,
 $R_2=H$, $R_3=CH_3$
 formononetin-7-*O*-beta-D-glucuronide(C17):
 $R_1=\text{glucuronosyl}$, $R_2=H$, $R_3=CH_3$
 calycosin(C13): $R_1=H$, $R_2=OH$, $R_3=CH_3$
 calycosin-7-*O*-beta-D-glucoside(C8): $R_1=\text{glucosyl}$,
 $R_2=OH$, $R_3=CH_3$
 calycosin-7-*O*-beta-D-glucuronide(C1):
 $R_1=\text{glucuronosyl}$, $R_2=OH$, $R_3=CH_3$
 calycosin sulphate(C18): R_1 or $R_3=SO_3H$
 daidzein-7-*O*-beta-D-glucuronide(C20):
 $R_1=\text{glucuronosyl}$, $R_2=R_3=H$



(6aR, 11aR)-3-hydroxy-9, 10-
 dimethoxypterocarpan(C15): $R=H$
 (6aR, 11aR)-3-hydroxy-9, 10-dimethoxypterocarpan-3-
O-beta-D-glucoside(C11): $R=\text{glucosyl}$
 (6aR, 11aR)-3-hydroxy-9, 10-dimethoxypterocarpan-3-
O-beta-D-sambubioside(C10): $R=\text{sambubiosyl}$
 (6aR, 11aR)-3-hydroxy-9, 10-dimethoxypterocarpan-3-
O-beta-D-glucoside-6''-*O*-malonate(C6): $R=6''\text{-O-}$
 malonylglucosyl
 (6aR, 11aR)-3-hydroxy-9, 10-dimethoxypterocarpan-3-
O-beta-D-glucuronide(C2): $R=\text{glucuronosyl}$



7, 2'-dihydroxy-3', 4'-dimethoxyisoflavan(C16):
 $R_1=R_2=R_3=H$, $R_3=R_4=CH_3$
 7, 3'-dihydroxy-2', 4'-dimethoxyisoflavan-7-*O*-beta-D-
 glucoside(C12): $R_1=\text{glucosyl}$, $R_2=R_4=CH_3$, $R_3=R_5=R_6=H$
 7, 2'-dihydroxy-3', 4'-dimethoxyisoflavan-7-*O*-beta-D-glucoside-
 6''-*O*-malonate(C7): $R_1=6''\text{-O-}$ malonylglucosyl, $R_2=R_5=R_6=H$,
 $R_3=R_4=CH_3$
 7, 2'-dihydroxy-3', 4'-dimethoxyisoflavan-2'-*O*-beta-D-
 glucuronide(C3): $R_2=\text{glucuronosyl}$, $R_1=R_5=R_6=H$, $R_3=R_4=CH_3$
 7, 2'-dihydroxy-3', 4', 5'-trimethoxyisoflavan-7-*O*-beta-D-
 glucuronide(C4): $R_1=\text{glucuronosyl}$, $R_2=R_6=H$, $R_3=R_4=CH_3$,
 $R_5=OCH_3$
 7, 2'-dihydroxy-3', 4'-dimethoxyisoflavan-7-*O*-beta-D-
 glucuronide(C5): $R_1=\text{glucuronosyl}$, $R_2=R_5=R_6=H$, $R_3=R_4=CH_3$
 7, 2'-dihydroxy-3', 4', 6'-trimethoxyisoflavan(C19):
 $R_1=R_2=R_3=H$, $R_3=R_4=CH_3$, $R_6=OCH_3$
 7, 3'-dihydroxy-2', 4'-dimethoxyisoflavan-7-*O*-beta-D-glucosyl-
 3'-*O*-beta-D-glucuronide(C21): $R_1=\text{glucosyl}$, $R_2=R_4=CH_3$,
 $R_3=\text{glucuronosyl}$, $R_5=R_6=H$

Fig. 4