

Pharmacokinetic Analyses of Ferulic Acid in Rat Plasma by Liquid Chromatography – Tandem Mass Spectrometry: A Synergistic Action of an Ancient Herbal Decoction Fo Shou San

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Abstract

Fo Shou San (FSS) is an ancient herbal decoction comprised of Chuanxiong Rhizoma (CR; Chuanxiong) and Angelicae Sinensis Radix (ASR; Danggui) in a ratio of 2: 3. FSS was first recorded by Xu Shuwei in Puji Benshi in Song Dynasty (AD 1132) of China, which was prescribed to treat women's ailments. Ferulic acid is one of the active chemicals in FSS decoction. By using ferulic acid as a marker chemical, determined by triple quadrupole tandem mass spectrometer, the pharmacokinetic properties of FSS were revealed here. In cultured human epithelial colorectal adenocarcinoma (Caco-2) cell monolayers, the absorption rate of ferulic acid under the herbal mixture was determined: the rate was markedly increased by over 80% as compared to ferulic acid alone. After oral administration of FSS, a quick absorption of ferulic acid was observed in rat plasma. The pharmacokinetic parameters indicated that the bioavailability and AUC (area under the curve) values of ferulic acid in FSS were determined. Our results showed that the membrane permeability and pharmacokinetic characteristics of ferulic acid were markedly enhanced in FSS, which partly explained the synergistic action of different herbs within a Chinese herbal decoction.

Keywords: Ferulic acid; Angelica sinensis; Chuanxiong rhizoma; TCM; Pharmacokinetics

Introduction

Traditional Chinese medicines (TCMs) have been used in clinical applications for many millennia. TCM plays an indispensable role in prevention and treatment of diseases. According to the old tradition, TCM is used as formulated water decoction that has a specific combination of different herbs, and which should be prepared by unique methodology to achieve specific efficacies. From the immense archives of herbal formulated decoction, Angelicae Sinensis Radix (ASR; Danggui; roots of Angelica sinensis (Oliv) Diels.) and Chuanxiong Rhizoma (CR; Chuanxiong; rhizomes of Ligusticum chuanxiong Hort.) are two commonly used herbs as a pair, and indeed they are considered as a "paired-herbs" according to Chinese medicinal theory. The combination of two herbs results in an herbal decoction named Fo Shou San (FSS) containing ASR and CR in a weight ratio of 3: 2. FSS, recorded by Xu Shuwei in Puji Benshi in Song Dynasty (AD 1132) of China, was prescribed to treat women's ailments, e.g. obstetric diseases, dystocia, vaginal bleeding with fetal movement, dead fetus in uterus and post-partum anemic fainting. Today, FSS is widely used to treat cerebral vascular and cardiovascular diseases, such as cerebral apoplexy, cerebral thrombosis and myocardial infarction [1,2]. According to TCM theory, the function of FSS is to "Nourish Blood" and "Promote Blood Circulation", i.e. functions in erythropoietic and circulatory systems, respectively. ASR and CR contain similar chemical composition, e.g. essential oils [3,4]. Besides essential oils, ferulic acid is the most abundant chemical within the two herbs. In different to essential oils, ferulic acid is highly stable in a water decoction.

Ferulic acid is one of the most abundant phenolic acids found in food, beverage, supplement and herbal medicine. It belongs to a family of plant hydroxycinnamic acids, including caffeic acid, sinapic acid and p-coumaric acid. Ferulic acid has various physiological functions. The structure of ferulic acid is based on a benzene ring, and similar to many phytochemicals, it acts as an antioxidant by absorbing free radicals from surrounding environment [5]. Many studies have documented

the beneficial properties of ferulic acid, including its strong antioxidant, free radical-scavenging and anti-inflammation activities [6-9]. Similar to other anti-oxidants, ferulic acid reduces the levels of cholesterol and triglyceride, thereby reducing the risk of heart disease [10]. Ferulic acid is often added as ingredient of anti-aging supplements. Studies have shown that ferulic acid can decrease blood glucose of diabetes patients [11]. In TCM, ferulic acid is an very important effective constituent of ASR and CR, i.e. having the action of "activating the blood circulation to dissipate blood stasis". The herbs containing ferulic acid are commonly used to treat ischemic heart diseases and stroke according to TCM theory [5]. Indeed, ferulic acid is one of the major components of FSS, and thus ferulic acid has been chosen for pharmacokinetic study of FSS. In our previous studies, the biological properties of FSS were shown in preventing platelet aggregation, stimulating erythropoiesis differentiation [12,13] and protecting endothelial functions [14]. Therefore, the results of this work will contribute to understand the pharmacokinetics information of ferulic acid in FSS and consequently the physiologic functions of ferulic acid in FSS in the prevention of diseases. Here, a sensitive and accurate quantification of low concentrations of ferulic acid in rat plasma by using HPLC-MS/MS was developed. The validated results showed a high sensitivity (with a LLOD as low as 0.5 ng/ml) and a small plasma volume (50 μ l) were required. This method was then successfully applied to reveal the

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role of FSS herbal mixture in affecting the metabolism of ferulic acid pharmacokinetic properties in rats after oral administration.

Materials and Method

Plant material and preparation of Fo Shou San

Fresh roots were obtained from China from September to October of 2008, listed as follows: 2-year-old ASR (roots of *A. sinensis*) from Minxian of Gansu province and 3-year-old CR (rhizomes of *L. chuanxiong*) from Guanxian of Sichuan province. These areas are known to produce the best quality of ASR and CR, respectively. The herbs were authenticated by one of the authors, Dr. Tina Dong. The corresponding vouchers as forms of whole plants, voucher # 08-11-1 for CR voucher # 08-9-1 for ASR, were deposited in Center for Chinese Medicine at The Hong Kong University of Science and Technology. In preparation of FSS, the amounts of crude drugs of ASR and CR were weighed according to a ratio of 3: 2 and then mixed well by vortexing, as in previous developed method [15]. The herbal mixture was boiled in 8 volumes of water (v/w) for one hour and extracted twice. For the second extraction of FSS, the residue from the first extraction was filtered before the process. The extracts were dried by lyophilization and stored at 4°C.

Chemical reagent

Ferulic acid was purchased from Sigma (St. Louis, MO, 99.9% purity) and docetaxel (internal standard, I.S., 99.5% purity) was provided by Shanghai Jinhe Bio-Technology Co., Ltd. (Shanghai, China). Acetonitrile of HPLC-grade and diethyl ether of analytical-grade were purchased from Merck (Darmstadt, Germany). Water (=18.2 MΩ) was obtained using an EASYPURE[®] II RF/UV ultra-pure water system (Barnstead International Corp. Dubuque, IA). Formic acid (HPLC-grade) was purchased from Dikma (Richmond Hill, NY) and high-purity nitrogen (99.999%) was used.

Preparation of ferulic acid standard and docetaxel solution

Accurately weighed 10 mg of ferulic acid, and which was dissolved in 5 ml of acetonitrile by using volumetric flask to make a stock solution of 200 mg/l. Then, a 4 mg/l ferulic acid solution was diluted and stocked in 4°C fridge for the following study. For the ferulic acid calibration curve, the working standard solutions were diluted by using acetonitrile-water (10:90, v/v), the concentrations were listed as follow: 2.5, 5, 25, 100, 500, 1000, 2000, 3000, 4000 ng/ml. For docetaxel solution, weighed 48.48 mg and dissolved it in 10 ml acetonitrile-water (10: 90, v/v) by using volumetric flask to make the concentration to 4.848 mg/ml. For the following assay, 48.48 µg/ml docetaxel solution was diluted by using acetonitrile-water (10:90, v/v), and the stock was stored in 4°C fridge.

Optimization of the condition of LC-MS/MS system

Analyses were acquired on an ACQUITY UPLC[™] system (Waters Corp, Milford, MA) with a cooling auto-sampler and column oven allowing accurate temperature control of the analytical column. An ACQUITY UPLC[™] BEH C₁₈ column (50 mm × 2.1 mm, 1.7 µm) was used. The chromatographic separations were accomplished using gradient elution with a mobile phase composed of acetonitrile (solvent A) and water (solvent B). The gradient conditions of the mobile phase were: 0 min 10% A, 0.5 min 50% A, 1.5 min 30% A, 2.5 min 70% A, 3.0 min 30% A and 3.5 min 10% A. The column temperature was maintained at 40°C with the flow rate set at 0.2 ml/min. The auto-sampler temperature was conditioned at 7°C. The injection volume was 5 µl using the partial loop mode for sample injection. The

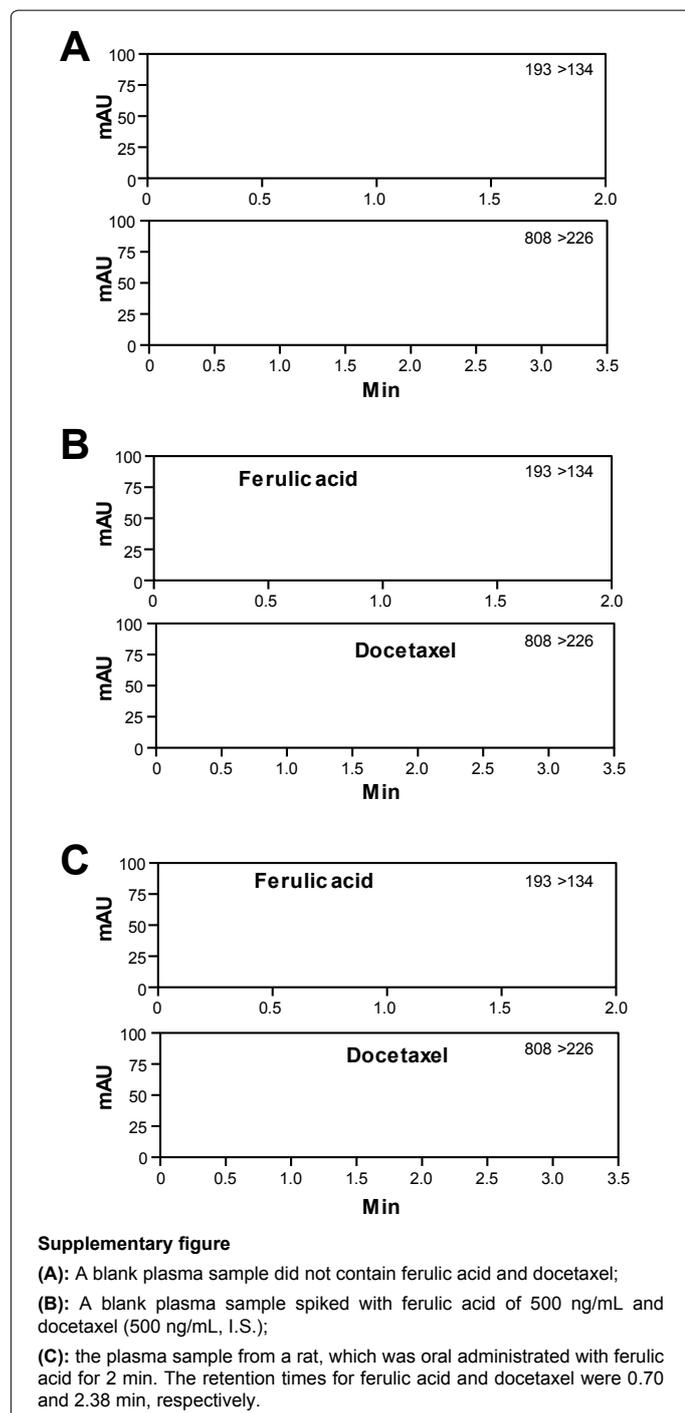
chromatographic run time per sample was 3.5 min. From 0.8 min to 3.5 min, the eluent was injected into the detector, and the remainder was diverted to waste. The separated compounds were detected by a Waters Tandem Quadrupole (TQ) Detector (Waters). The mass spectrometer was operated with electrospray ionization (ESI) interface in negative and positive ionization mode for ferulic acid and I.S., respectively. In negative mode for ferulic acid, the ionization source conditions were: capillary voltage of 3.2 kV, cone voltage of 32 V, the optimized collision energy was 19 V; source temperature 120°C and desolvation temperature 350°C. The cone and desolvation gas flow rates were 50 l/hour and 600 l/hour, respectively. Under these HPLC-MS/MS conditions, the chemicals were analyzed by multiple reaction monitoring (MRM) of the transitions of m/z 193→134 for ferulic acid and m/z 808→226 for docetaxel, respectively. The scan time was set at 0.2 sec per transition. A standard solution of ferulic acid and docetaxel was directly infused with the mobile phase into the mass spectrometer with ESI as the ionization source. In the ion full-scan spectra, the most abundant ions were deprotonated molecules [M-H]⁻ m/z 193 for ferulic acid and protonated molecules [M+H]⁺ m/z 808 for docetaxel, respectively. Parameters, such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas, were optimized to obtain the highest intensity of deprotonated and protonated molecules. The method validation was fully described in [16,17].

Stability of ferulic acid standard solution in rat plasma

For the preparation of rat plasma sample, 50 µl of plasma were mixed together with 50 µl of mobile phase, 50 µl of 484.8 ng/ml docetaxel solutions and 0.5% formic acid 20 µl by vortexing. After adding 3 ml diethyl ether, the solution was mixed by vortexing for 1 min. Then, the mixture was centrifuged at 3000 × g for 10 min. The organic fraction of supernatant was obtained, which was then dried by applying 40°C nitrogen. This dried product was dissolved in 100 µl of the mobile phase solution to get the plasma sample. First of all, the chromatography of blank sample was shown in Supplementary figure A, which was the rat plasma without adding ferulic acid or docetaxel. Supplementary figure B shows the chromatography of ferulic acid and docetaxel standard solution. The retention time of ferulic acid was at 0.7 min, while the retention time of docetaxel was at 2.38 min. Supplementary figure C showed the chromatography of ferulic acid and docetaxel with the oral administration of rats. These results suggested that docetaxel could be used as an I.S. in rats, which would not affect the analysis of ferulic acid.

Pharmacokinetic study

The developed method was used to determine the plasma concentrations of ferulic acid in six healthy adult male Wistar rats (Laboratory Animal Center of Shenyang Pharmaceutical University, Shenyang, Liaoning, China) weighing 220 ± 20 g (mean ± standard deviation, SD). Before the day of given drugs, the rats were fasted 24 h but were allowed water ad libitum. The animal studies were approved by the Shenyang Pharmaceutical University Animal Care and Use Committee. The rats were intragastrically given FSS (5 mg/kg) and ferulic acid (1.5 mg/kg), and then 0.25 ml blood samples were collected into heparinized tubes according to the following time schedule: 0, 2, 5, 10, 15, 20, 30, 40, 60, 90, 180 and 360 min post-dosing. The blood samples were centrifuged immediately at 3500 rpm for 10 min to obtain plasma. The plasma samples were labeled and kept frozen at -20°C until analysis [16,17]. All animal studies were conducted in accordance with regulations of Experimental Animal Administration issued by The State Committee of Science and Technology of People's Republic of China on November 14th, 1988, as well as the related ethics regulations of Shenyang Pharmaceutical University.



Trans-epithelial permeability of ferulic acid

Caco-2 cells were grown in DMEM, supplemented with 20% FBS, 1 mM nonessential amino acids, 100 units/ml penicillin and 100 units/ml streptomycin in a humidified CO₂ (5%) incubator at 37°C. After reaching 80% confluence, Caco-2 cells were harvested with 0.25% trypsin–EDTA solution. For the intestinal permeability experiments, Caco-2 cells (passage 45–55) were seeded in 12-well Transwell inserts (Millipore Corp., Bedford, MA) at seeding density of 5×10⁵ cells/cm² [18]. In the inserts, there were polyester filter membranes with a pore size of 0.4 μm and a surface area of 1.12 cm². Culture medium was

replaced every other day for the first 14 days and daily thereafter for the next 7 days until the monolayer expressed properties that closely resemble morphologic and functional characteristics of normal enterocyte.

The cell monolayers were considered tight enough for the transport experiments. The P_{app} (cm/s) values were calculated according to the following equation [19]:

$$P_{app} = \left(\frac{dQ}{dt} \right) \times \frac{1}{C_0} \times \frac{1}{A}$$

Where: dQ/dt was the slope of the cumulative concentration of the compound in the receiving chamber over time; C₀ represented the initial concentration in the donor side; and A was the surface area of the porous membrane (A = 1.12 cm²).

All the studies were conducted at 37°C. Prior to the experiment, the inserts were washed twice and equilibrated for 30 min with pre-warmed Hank's balanced salt solution (HBSS, pH 7.4). Transepithelial permeability of ferulic acid was measured by RRLC-QQQ-MS/MS. Transport studies were conducted in the absorptive direction (apical→basolateral or basolateral→apical). The transport buffer containing test compounds was added on the apical (0.5 ml), while the basolateral (1.5 ml) side of the inserts contained the corresponding volume of HBSS. At the incubation of 120 min, aliquot of 100 μl was withdrawn from the receiver chambers and was immediately replenished with an equal volume of pre-warmed HBSS [20,21]. The concentrations of the tested compounds in the transport medium were immediately analyzed by RRLC-QQQ-MS/MS.

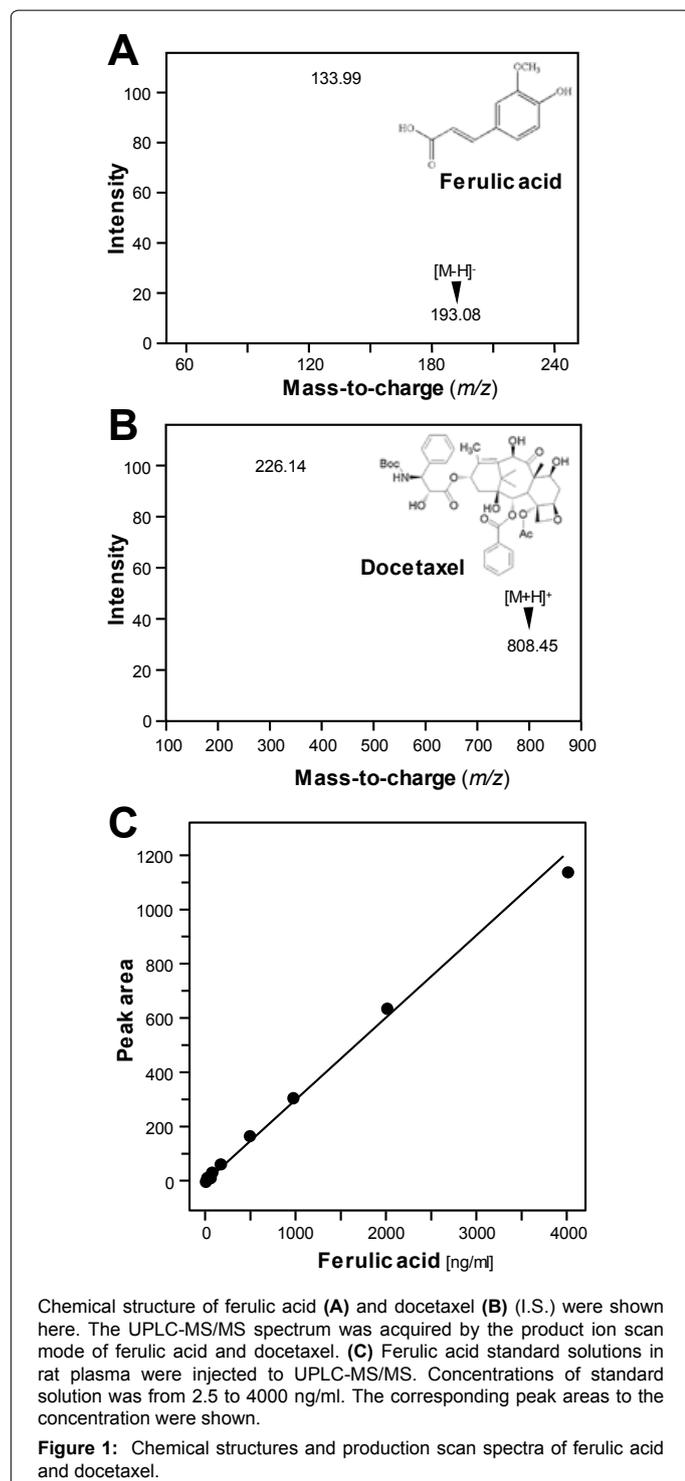
Result

Optimization of the condition of LC-MS/MS system

The product ion scan spectra showed high abundance fragment ions at m/z 134 and 226 for ferulic acid and docetaxel, respectively. The product-ion spectra and structures of these compounds are shown in Figure 1. The collision gas pressure and collision energy of collision-induced decomposition (CID) were optimized for maximum response of fragmentation of m/z 193 for ferulic acid and m/z 808 for docetaxel. The ion transitions of m/z 193→134 for ferulic acid and m/z 808→226 for docetaxel were chosen for MRM. The chromatographs of blank sample, ferulic acid and docetaxel were shown in Supplementary figure. The retention time of ferulic acid was at 0.7 min, while the retention time of docetaxel was at 2.38 min, which was not altered even after the oral administration in rats (Supplementary figure). These results suggested that docetaxel could be used as an I.S. in rats, which did not affect the analysis of ferulic acid. In UPLC-MS/MS quantification, ferulic acid in rat plasma was analyzed. In order to develop a calibration curve, different concentrations of ferulic acid from 2.5 to 4000 ng/mL was made in rat plasma. The equation of ferulic acid in rat plasma here was: $y=0.3477x + 4.4147$ ($r^2=0.9937$). The LOD and LOQ of ferulic acid standard solution in rat plasma were determined, which were 0.5 and 2.5 ng/mL, respectively. These validating parameters were also applied for ferulic acid in cultured medium, as in [16,17].

Calibration curve of ferulic acid, LOQ, precision and recovery of ferulic acid and docetaxel standard solution in rat plasma

In UPLC-MS/MS quantization, ferulic acid standard solution in rat plasma was analyzed. In order to develop the calibration curve of ferulic acid, different concentrations of ferulic acid: 2.5, 5, 25, 100, 500, 1000, 2000 and 4000 ng/ml was made in rat plasma. Table 1 shows the calibration curve of ferulic acid standard solution. The linearity of



ferulic acid was from 2.5 to 4,000 ng/ml by testing with UPLC-MS/MS. The equation of ferulic acid in rat plasma here was: $y=0.3477x + 4.4147$ ($r^2=0.9937$). The LOD and LOQ of ferulic acid standard solution in rat plasma were determined, which was 0.5 and 2.5 ng/ml, respectively. Figure 2 shows the calibration curve of ferulic acid standard solution in rat plasma by testing with an UPLC-MS/MS.

In order to test the variations of ferulic acid solutions in rat plasma, standard solution of ferulic acid at a concentration of 2.5 ng/

ml was analyzed. The precision of 6 analytes were shown in Table 2. The RSD of overall variations was 15.3% for six analytes. These results proved that the precision of ferulic acid was good. In order to test the intra- and inter-day variation, different concentrations of ferulic acid standard solution was analyzed on three different days. The precision and repeatability of six analytes from each concentration were shown in Table 3. These data indicated that the RSD of overall intra- and inter-day variations were less than 16.47% for three different concentration of ferulic acid, and the LOQ of 2.5 ng/ml of ferulic acid in rat plasma was achieved. Besides, the validation studies of this method proved that this assay had good repeatability with RSD less than 12.32% for three different concentrations. For recovery test of ferulic acid standard solution, three different concentrations of ferulic acid (5, 500 and 3,000 ng/ml) were added with the same amount of docetaxel (500 ng/ml), as to calculate the recovery rate. The recovery rate of ferulic acid at 5 ng/ml, 500 ng/ml and 3000 ng/ml were ~83.6%, ~75.8% and ~72.5%, respectively (Table 3); the recovery rate of internal standard, docetaxel, was ~88.2%. These results indicated that the recovery rate of ferulic acid and docetaxel were good by testing with UPLC-MS/MS system under the previously described mobile phase conditions.

During the experiment, the ferulic acid in rat plasma would be freeze and thaw for several times, or the samples would be stored in long-term cold fridges. Therefore, the stability of samples was our concern. In order to evaluate the stability of ferulic acid in rat plasma after incubation in room temperature, or freeze-thaw cycles or long-term cold storage, three replicates of ferulic acid standard solution in rat plasma at different concentrations (5, 500 and 3,000 ng/ml) were subjected to different conditions to test the stability of samples. Step 1, the ferulic acid standard solutions in rat plasma were incubated at room temperature for 4 hours, the stability of ferulic acid was tested by comparing the mean values of each concentrations of ferulic acid standard solution in rat plasma to the added ones (Table 4). These data indicated that the RSD were less than 9.1% for the three analytes. Step 2, after the samples were incubated in room temperature for 4 hours (step 1 procedure), the samples were then stored at -20°C for 12 hours, repeated step 1 and step 2 three times to mimic the freeze-thaw cycles during the experiments. The stability of ferulic acid standard solution in rat plasma in different concentrations was accessed by comparing the mean concentration of three freeze-thaw cycled with the concentration of each freshly prepared standard solutions (Table 5). These data indicated that the RSD were less than 8.4% for all three analytes. Then,

Chemical	Calibration curve ^a	Correlation coefficient (r^2)	Linear range (ng/ml)	LOD ^b (ng/ml)	LOQ ^c (ng/ml)
Ferulic Acid	$y=0.3477x + 4.4147$	0.9937	2.5-4000	0.5	2.5

^a The calibration curve was constructed by plotting the peak area versus each concentration. The calibration curve was derived from three data points, $n = 3$, and the SD was < 5% of the Mean.

^b LOD refers to the limits of detection

^c LOQ refers to the limits of quantification.

Table 1: Calibration curves of ferulic acid standard solution in rat plasma were determined by using UPLC-MS/MS method during method validation.

Chemical	Spiked (ng/ml)	Precision			
		Mean (ng/ml)	SD	RSD (%)	RE ^a (%)
Ferulic Acid	2.5	2.47	0.38	15.3	-1.43

Ferulic acid at the concentration of 2.5 ng/ml in rat plasma was prepared and then injected into UPLC-MS/MS to determine the precision. Mean of the concentration (ng/ml) of ferulic acid in rat plasma, SD, RSD (%) and RE (%) was calculated, $n = 6$. ^a refers to relative errors.

Table 2: Precision and accuracy at the LOQ of 2.5 ng/ml for ferulic acid in rat plasma determined by UPLC-MS/MS method.

Ferulic acid Added (ng/ml)	Precision				Repeatability (n = 6)		Recovery ^a (n = 3)	
	Intra-day (n=6) ^b		Inter-day (n=6) ^c		Mean (ng/ml)	RSD (%)	Mean (%)	RSD (%)
	Mean (ng/ml)	RSD (%)	Mean (ng/ml)	RSD (%)				
5	5.31	16.47	5.12	13.31	5.17	12.32	83.6	9.31
500	452.71	2.33	470.44	0.75	488.85	8.67	75.8	1.55
3000	2688.49	3.83	2988.17	3.32	2989.05	8.94	72.5	0.81
Docetaxel (I.S.) (500ng/ml)	-	-	-	-	-	-	88.2	2.14

Ferulic acid at different concentrations of 5, 500, 3000 ng/ml in rat plasma were prepared and then injected into UPLC-MS/MS. Mean of the each concentration (ng/mL) of ferulic acid in rat plasma, RSD (%) and RE (%) was calculated, n = 3.

^a Recovery (%) = 100 X (amount found-original amount)/ amount spiked. The data was presented as average of three independent determinations.

^b The intra-day analysis refers to the sample examined for six replicates within one day.

^c The inter-day analysis refers to the sample examined in duplicates over three.

Table 3: Precision and accuracy of the UPLC-MS/MS method to determine ferulic acid in rats plasma (n=3 days, six replicates per day).

Ferulic acid Added (ng/ml)	Stability			
	Mean (ng/ml)	SD	RSD (%)	RE (%)
5	5.09	0.46	9.1	1.9
500	432.51	9.80	2.3	-13.5
3000	3084.35	77.39	2.5	2.8

Ferulic acid standard solution at different concentrations of 5, 500 and 3,000 ng/ml in rat plasma was prepared, and then incubated at room temperature for 4 hours. The samples were then injected into UPLC-MS/MS to determine the stability of ferulic acid. Mean of the concentration (ng/ml) of ferulic acid in rat plasma, SD, RSD (%) and RE (%) was calculated, n = 3.

Table 4: Stability of the analysis of ferulic acid incubated in room temperature for 4 hours.

the samples were post-treated in reconstituted extracts and stored at 7°C for 12 hours, the samples were then tested by using UPLC-MS/MS. The stability of samples was shown in Table 6, which indicated that the RSD was less than 4.0%. Also, the ferulic standard solutions in rat plasma at different concentration (5, 500 and 3,000 ng/ml) were stored at -20°C for 30 days. The stability of ferulic acid standard solution in rat plasma was accessed by comparing the mean concentration of the stored samples with the concentration of each freshly prepared standard solution (Table 7). The RSD was less than 4.9% after storage. In summary, ferulic acid was stable in rat plasma under the above mentioned conditions, which mimicked the experimental conditions.

Pharmacokinetic study of ferulic acid in rat plasma after oral administration of Fo Shou San

The pharmacokinetic studies of ferulic acid have been determined previously [16,17]. Here, we analyzed the comparative study of pharmacokinetic parameters of ferulic acid after oral administration of ferulic acid alone and FSS. The developed method, described previously, was used to determine the plasma concentrations of ferulic acid in rats. Five g/kg of FSS, contained ~25 mg/kg of ferulic acid, were orally administrated to the fasted-rats; 1.5 mg/kg of ferulic acid were also orally administrated to the fasted rats [16]. Serial blood samples were collected at pre-determined time points from 2 to 360 min after oral administration. The profile of mean plasma concentration of ferulic acid versus the time was shown in Figure 3. After oral administration of FSS, a quick maximal absorption of ferulic acid was observed, i.e. known as T_{max} value at ~10 min (Figure 2): this was quite similar to the T_{max} value of oral administration of ferulic acid alone (Figure 2 and Table 8). The C_{max} of ferulic acid: 504.2 ng/ml and 1458.70 ng/ml were observed after the intake of ferulic acid (1.5 mg/kg) and FSS (5 g/kg), respectively (Table 8). An intravenous injection of ferulic acid (1 mg/kg) was served as a control for further comparison: the pharmacokinetic parameters achieved for ferulic acid were rather similar to oral administration (Table 8). The AUC of plasma concentration of ferulic acid alone and ferulic acid in FSS was determined after oral administration (Figure 2). Moreover, the bioavailability (BA) was also determined as shown in

Table 8. The relative BA was 42.93%, where the absolute BA was 61.07% (Table 8).

Effects of Fo Shou San on membrane permeability of ferulic acid across Caco-2 cell monolayers

Caco-2 cell line, derived from a colon carcinoma, was cultured under specific condition, and which could be differentiated and polarized to have morphological and functional characteristics of mature small-intestinal enterocytes forming a monolayer. This monolayer was shown to be a valuable tool in predicting human intestinal permeability [18,22,23]. The polarized monolayer expresses small intestinal hydrolase activities with microvilli on the apical side, including sucrose-isomaltase, lactase, aminopeptidases [22]. Caco-2 monolayers (Figure 3), growing on permeable filters, formed tight junctions and expressed transporters on the apical and basolateral surfaces [24]. In order to compare the P_{app} value between ferulic acid alone and that in herbal extracts, Caco-2 monolayers were treated with ferulic acid and herbal extracts. Then, the intestinal permeability of ferulic acid was evaluated. The P_{app} values for apical to basolateral flux of ferulic acid alone or ferulic acid in herbal extracts were above 10^{-6} cm/s (Figure 3). The efflux ratios of ferulic acid in FSS were about ~1.58, suggesting greater permeability in apical to basolateral (Figure 3), which is slightly higher than that of ferulic acid alone of ~0.92. Since passive diffusion and carrier-mediated flux are known to be two major pathways for a molecule to permeate across the intestinal epithelium [25], these results indicated that ferulic acid could permeate across Caco-2 monolayer via passive diffusion mechanisms. Also, the efflux ratio of ferulic acid in FSS was higher than that of ferulic acid alone suggested that the ingredients of FSS could induce the membrane permeability of ferulic acid.

Conclusion

In clinical practice, the majority of TCM prescriptions are composed of different herbs. The paired-herbs are used together mainly because the action of a single herb is usually very limited. The purpose of TCM paired-herbs is to produce a synergistic action to

Ferulic acid Added (ng/ml)	Stability			
	Mean (ng/ml)	SD	RSD (%)	RE (%)
5	5.17	0.44	8.4	3.4
500	518.49	12.13	2.3	3.7
3000	2789.28	120.79	4.3	-7.0

Ferulic acid standard solution at different concentrations of 5, 500 and 3,000 ng/ml in rat plasma was prepared. After three freeze-thaw cycles, the samples were injected into UPLC-MS/MS to determine the stability of ferulic acid. Mean of the concentration (ng/ml) of ferulic acid in rat plasma, SD, RSD (%) and RE (%) was calculated, $n = 3$.

Table 5. Stability of the analysis of ferulic acid after freeze-thaw cycles.

Ferulic acid Added (ng/ml)	Stability			
	Mean (ng/ml)	SD	RSD (%)	RE (%)
5	5.05	0.20	4.0	1.0
500	432.54	1.81	0.4	-13.5
3000	3064.17	89.48	2.9	2.1

Ferulic acid standard solution at different concentrations of 5, 500 and 3,000 ng/ml in rat plasma was prepared. After post-treatment for 12 hours at 7°C, the samples were injected into UPLC-MS/MS to determine the stability of ferulic acid. Mean of the concentration (ng/ml) of ferulic acid in rat plasma, SD, RSD (%) and RE (%) was calculated, $n = 3$.

Table 6: Stability for the analysis of ferulic acid after post-treatment for 12 hours (7°C).

Ferulic acid Added (ng/ml)	Stability			
	Mean (ng/ml)	SD	RSD (%)	RE (%)
5	5.46	0.27	4.9	9.1
500	565.09	4.10	0.7	13.0
3000	2789.91	41.77	1.5	-7.0

Ferulic acid standard solution at different concentrations of 5, 500 and 3,000 ng/ml in rat plasma was prepared. After storage for 30 days at -20°C, the samples were injected into UPLC-MS/MS to determine the stability of ferulic acid. Mean of the concentration (ng/ml) of ferulic acid in rat plasma, SD, RSD (%) and RE (%) was calculated, $n = 3$.

Table 7: Stability for the analysis of ferulic acid after storage at -20°C for 30 days

Parameters	Ferulic acid ^a	Ferulic acid ^b	FSS ^c
	(1 mg/kg)	(1.5 mg/kg)	(5 g/kg)
C_{max} (ng/mL)	948.00 ± 73.00	602.40 ± 111.80	1846.20 ± 533.14
t_{max} (h)	----	0.08 ± 0.00	0.09 ± 0.05
$t_{1/2}$ (h)	1.65 ± 0.53	2.59 ± 0.44	1.82 ± 1.09
Ke (L/h)	0.45 ± 0.13	0.28 ± 0.05	0.38 ± 0.12
AUC_{0-t} (ng·h/mL)	123.10 ± 12.18	129.80 ± 28.40	1007.86 ± 262.52
$AUC_{0-∞}$ (ng·h/mL)	128.40 ± 8.93	144.50 ± 27.40	1211.08 ± 431.22
Relative BA		42.93% ^d	
Absolute BA	61.07% ^e		

The rats were oral administrated with FSS (5 g/kg) or ferulic acid (1.5 mg/kg), or intravenous injection of ferulic acid (1 mg/kg), the blood samples were collected according to the different time points from 2 to 360 min. The plasma were subjected to UPLC-MS/MS determination of different pharmacokinetic parameters (as in Figure 3), including T_{max} , C_{max} , $T_{1/2}$, Ke, AUC_{0-t} and $AUC_{0-∞}$ of ferulic acid. Values are expressed in Mean ± SEM, $n = 6$.

^a A single intravenous of ferulic acid (1 mg/kg);

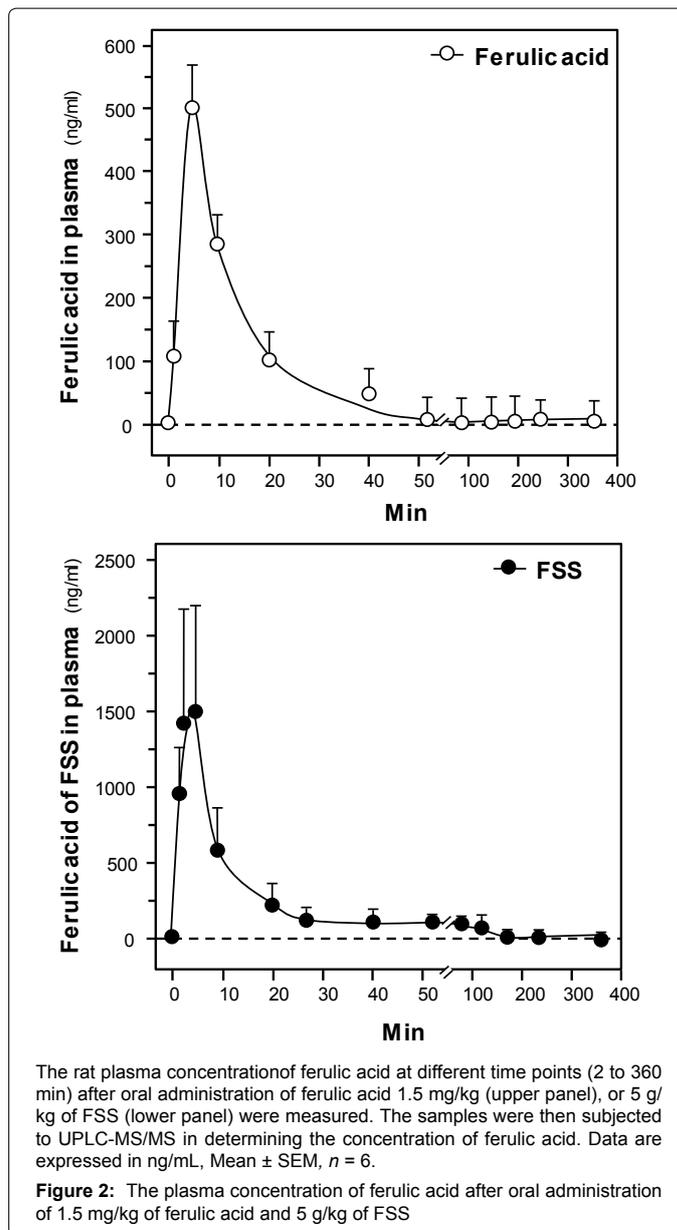
^b Oral administration of ferulic acid (1.5 mg/kg);

^c Oral administration of FSS (5 g/kg).

^d As compared to FSS^c, use AUC_{0-t} to calculate the relative BA of ferulic acid.

^e As compared to FSS^c, use AUC_{0-t} to calculate the absolute BA of ferulic acid.

Table 8: The main pharmacokinetic parameter of ferulic acid after oral administration of FSS

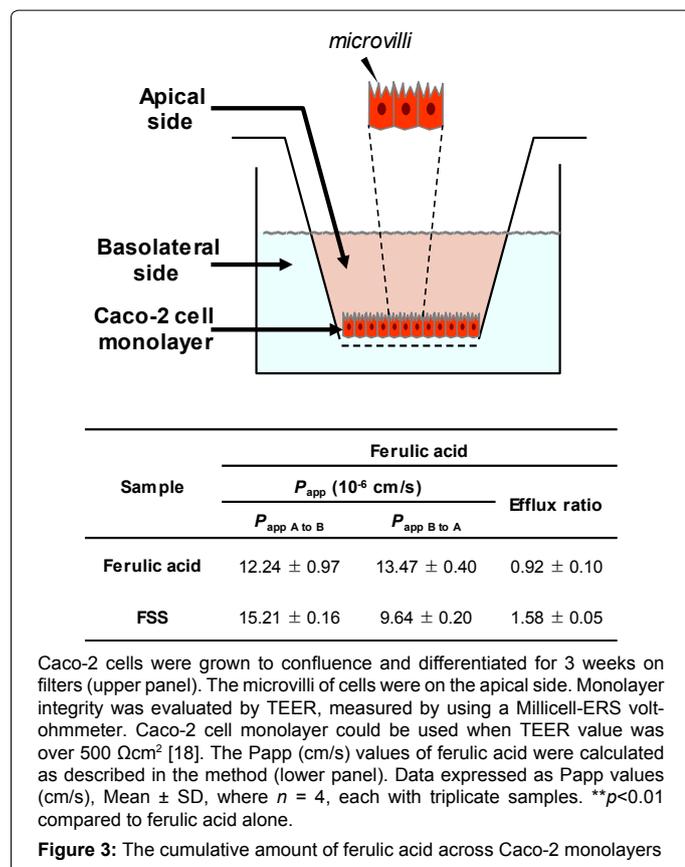


The rat plasma concentration of ferulic acid at different time points (2 to 360 min) after oral administration of ferulic acid 1.5 mg/kg (upper panel), or 5 g/kg of FSS (lower panel) were measured. The samples were then subjected to UPLC-MS/MS in determining the concentration of ferulic acid. Data are expressed in ng/mL, Mean ± SEM, $n = 6$.

Figure 2: The plasma concentration of ferulic acid after oral administration of 1.5 mg/kg of ferulic acid and 5 g/kg of FSS

enhance therapeutic efficacy and/or to eliminate toxicity and adverse effects. The paired-herbs can be used not only in a distinct formula, but also together with different herbs in a complex TCM decoction. Among all the paired-herbs, ASR and CR are the most popular pair. By statistics, there are more than 150 TCM formulae that contain CR and ASR together. Also, ASR and CR could be used together as FSS. However, the molecular action mechanisms of compatibility of paired-herbs have never been determined. Therefore, we chose FSS as a study mode here, even though the active ingredient within FSS was not been determined yet. In our previous studies, we have set up systematic approaches to standardize FSS: (i) development of quality control of FSS [12]; (ii) verification of the biological functions of FSS [12-14]; (iii) investigation of the molecular mechanisms of FSS [14].

Ferulic acid is beneficial to health due to its activities against coronary heart disease, thrombosis, carcinogenesis and mutagenesis, and which is one of main components of FSS decoction. To understand the physiological functions of FSS, it is necessary to investigate its



metabolism. Therefore, ferulic acid was chosen to study here for the absorption of FSS after oral administration. Ferulic acid is soluble in organic solvents, and thus which could easily go through biological membrane. From the concentration-time curve, the pharmacokinetic profile of ferulic acid in FSS decoction was determined. The C_{max} reached at 5 min after oral administration, which suggested that ferulic acid was absorbed freely in the gut. The pharmacokinetic studies on various herbal decoctions in animals have revealed the BA of many active ingredients could show delay absorption and elimination as to maintain a longer turnover within the blood circulation, e.g. higher C_{max} and AUC_{0-lim} of baicalin, wogonoside and ligustrin in rat after administration of Huangqin Tang decoction [26].

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