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LC/MS/MS determination and pharmacokinetic studies of six compounds in rat plasma following oral administration of the single and combined extracts of *Eucommia ulmoides* and *Dipsacus asperoides*

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[ABSTRACT]

AIM: To establish and apply a new LC/MS/MS method for the simultaneous, quantitative determination of six ingredients, aucubin (AU), geniposide (GP), geniposidic acid (GPA), pinoresinol diglucoside (PDG), secologanin (SLG), and loganin (LG) in single and combined extracts of *Eucommia ulmoides* and *Dipsacus asperoides*.

METHOD: Using the LC/MS/MS-ESI$^-$-MRM mode to detect the six compounds, chromatographic separation was achieved on an Agilent Eclipse plus C_{18} column, and the mobile phase consisted of solvent A (CH_3CN) and solvent B (H_2O containing 0.01% CH_3COOH *V*/*V*).

RESULTS: This method was successfully applied to quantify the six compounds in rat plasma after oral administration, and showed good precision, accuracy, reproducibility, and linear regression ($r^2 > 0.99$).

CONCLUSION: The results showed that following the use of the two medicinal plants, for AU and GP, the values of t_{max} markedly increased, and the values of c_{max} markedly decreased. It was found that the compatibility of the medicinal plants might affect their pharmacokinetic properties of their constituents.

[KEY WORDS] Pharmacokinetics; Compatibility; *Eucommia ulmoides*; *Dipsacus asperoides*; LC/MS/MS

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Introduction

Compound prescriptions composed of multiple ingredients are commonly used in traditional Chinese medicine (TCM). Medicinal plant compatibility in TCM may affect the active or toxic compounds' therapeutic and/or the toxic effects of TCM prescriptions [1], as the plant drug-plant drug interactions might influence the *in vivo* pharmacokinetic properties of some components in the prescriptions [2-6]. These may affect the absorption, distribution, metabolism, and/or excretion, especially of the main active and toxic components, because their pharmacokinetic properties usually affect the efficacy and/or side effects of the whole prescription. In the clinic, multi-component compatibility is also used in some special diseases. By fine-tuning the correct mixture of medicinal plants, Sawyers *et al* found that specific molecular targeting might prevent the growth of tumor cells [7], and De Francesco *et al.* reported that combinations of multiple agents could be successful to treat chronic HCV infection [8]. Thus, research of multi-component compatibility is very important and necessary.

Duzhong Wan is a traditional Chinese medicine mainly used to treat the low back pain of pregnancy. Its major components are

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Eucommia ulmoides Oliv. (Eucommiaceae) and *Dipsacus asperoides* C.Y Cheng & Ai (Caprifoliaceae) (crude drug ratio 1:1), which are a commonly used, compatible medicinal plant pair in Chinese traditional medicine. Several parts of *E. ulmoides* are reported to have a variety of pharmacologic actions. Water-soluble alkaloids of its male flowers had significant sedative and hypnotic effects in mice [9], the polyphenolic compounds of its cortex extract prevented OVX-induced osteoporosis [10], its leaf extract might be a potential source of natural antioxidants [11], and the compounds of its leaf extract were reported to exhibit anti-obesity properties in rat models of metabolic syndrome [12]. The antihypertensive ingredients, including geniposide, geniposide acid, and so on in *Eucommia* were reported to produce significant dilation of the chest artery of SD rats [13]. *D. asperoides* is often used for the treatment of bone diseases, and Jung *et al* found that an aqueous extract of Dipsaci radix had anti-inflammatory and anti-arthritic effects in arthritic mice [14].

The pharmacodynamic effects of plant medicines are usually attributed to the synergistic effects of their various phytochemical constituents, and the analysis of their active components is a key step to understanding their effects and the underlying mechanisms. Therefore, it is necessary to study the exposure to these ingredients *in vivo*. Wang *et al.* established a sensitive HPLC/MS/MS method for the pharmacokinetic study of pinoresinol diglucoside in rat plasma following the oral administration of *E. ulmoides* extract [15]. A rapid and sensitive HPLC-ESI-MS method was established by Li *et al* to determine the asperosaponin VI content in rat plasma following the oral administration of asperosaponin VI, and they found double peaks in its plasma concentration curve, which might be related to enterohepatic recirculation [16]. Han *et al*. established a UPLC/ESI-QTOF-MS method to analyze the metabolites of geniposide and ten metabolites were found in rat urine following oral administration [17]. Zhou *et al*. found that akebia saponin D, a main compound in *E. ulmoides*, had very low oral bioavailability, to poor transepithelial permeability and to being a substrate of MRPs efflux transporters in the intestine [18]. Although pharmacokinetic studies abound on *E. ulmoides* and *D. asperoides*, most were based on single compounds. In this study, a sensitive and rapid LC/MS/MS analytical method was developed to study the changes in drug pharmacokinetics *in vivo*. The other objective of this research was to reveal the *in vivo* effects of combination on each ingredient in the TCM prescription changes, as $AUC_{0\text{-}24}$, $Auc_{0\text{-}\infty}$, t_{max}, C_{max}, $t_{1/2}$, and so on.

Materials and Methods

Materials and chemicals

Purified aucubin (AU), geniposide (GP), geniposidic acid (GPA), and pinoresinol diglucoside (PDG) were obtained from the laboratory of Prof. SU YF, Tianjin University (Tianjin, China), secologanin (SLG) and loganin (LG) were produced in this laboratory. The compounds had purities ⩾98%, and their structures are shown in Fig. 1.

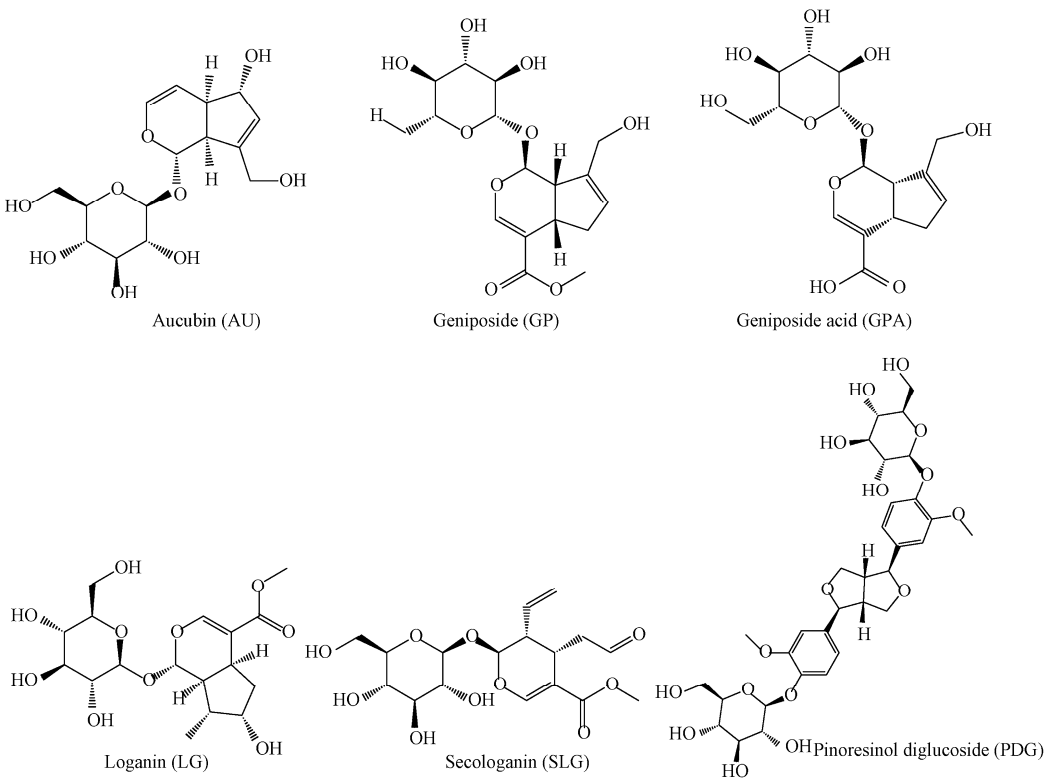

Fig. 1 Structures of the six compounds analyzed in this study

E. ulmoides and *D. asperoides* were purchased from the Corporation of Medicinal Herbs and Extracted Granule of Qixing (Purchased from Sichuan,China, and identified by Tianxiang Li, an associate professor of Tianjin university of TCM. The voucher accession numbers of *E. ulmoides* and *D. asperoides* were *1004052* and *100674*, respectively.). Reagents used were HPLC grade acetonitrile and methanol were purchased from Fisher (Fisher Chemical, (Fair Lawn, New Jersey 07410, USA), while acetic acid was purchased from Tedia (1000 Tedia Way, Fairfield, OH 45014, USA). Pure water was prepared with a Milli-Q system operating at 18.2 $mol \cdot L^{-1}$ (Millipore, Bedford, MA, USA).

General procedure and instrument condition

A validated bioanalytical method, capable of measuring AU, GP, GPA, PDG, SLG, and LG in plasma at the same time, was established based on LC/MS/MS.

The LC/MS/MS system consisted of an Agilent 6430 Quad LC/MS/MS (QQQ) equipped with electro-spray ionization (ESI) source (Agilent, Santa Clara, CA USA), coupled with Agilent MassHunter acquisition software version B.03.01 and analysis workstation software version B.03.02/Build 3.2.170.25, and an Agilent 1200 model (Agilent, Santa Clara, CA, USA). The chromatographic separation was achieved on an Agilent Eclipse plus C_{18} (2.1 mm × 150 mm, 5 μm; Agilent Co., Santa Clara, CA, USA). The mobile phase consisted of solvent A (CH_3CN) and solvent B (H_2O containing 0.01% CH_3COOH *V*/*V*). The elution schedule, which included seven segments, was as follows: 95%–50% B from 0 to 3 min, 50%–20% B from 3 to 7 min, 20%–5% B from 7 to 9 min, 5%–5% B from 9 to 14 min, 5%–30% B from 14 to 15 min, 30%–95% B from 15 to 18 min, 95% B from 18 to 20 min, and post-run of 3 min for column equilibration. The flow rate was 0.3 $mL \cdot min^{-1}$, and the temperature was kept at 20 °C throughout the period of analysis.

The MS/MS analysis was conducted using multiple reaction monitoring in the negative-ion electro-spray ionization mode for AU, GP, GPA, PDG, SL,G and LG using the transitions *m/z* 405.0→183.1, 447.0→225.0, 373.0→122.8, 681.3→357.2, 417.1→195.0, and 449.2→227.1, respectively. The ESI source of drying gas was set at the flow rate of 9.0 $L \cdot min^{-1}$, using a temperature of 350 °C and capillary tube voltage of 4 000 V. The plasma PK parameters were estimated by a non-compartmental method using Drug and Statistics (DAS) software version 1.0.

Preparation of crude drug extract

Extraction of the bark of E. ulmoides

The bark of *E. ulmoides* was cut into slices, decocted twice with 70% ethanol for two hours using ten times the solvent each time. The decoctions were combined, concentrated to a relative density of 1.2–1.3 at 90 °C, and then stored at −15 °C pending use. The extraction recovery was 13.5%. Using the conditions described above, the contents of the four main ingredients in the extract, AU, GP, GPA, and PDG, were 19.45, 2 786.68, 7 535.55, and 9 025.44 $ng \cdot mg^{-1}$, respectively.

Extraction of the root of D. asperoides

The root of *D. asperoides* was extracted using the same method used for bark of the *E. ulmoides*, and its extraction recovery was 30.8%. Using the conditions described above to analyze the extract, the contents of the two main ingredients, SLG and LG, in the extract were 13 032.49 and 2 725.18 $ng \cdot mg^{-1}$, respectively.

Preparation of standard solutions

The six standard compounds were combined to make up the mixed standard solution using methanol, in which every ingredient was present at 20 $\mu g \cdot mL^{-1}$. The stock solution was stored at –20 °C until use.

Method validation

Calibration curves and low limit of quantitation

The stock solution was diluted with methanol to obtain a series of standard solutions containing 10, 40, 100, 200, 400, 1 000, 2 000, and 4 000 $ng \cdot mg^{-1}$ of the six standard components. Then the diluted solutions were pipetted into 1 mL centrifuge tubes and mixed to prepare eight concentrations of standard serum samples (1, 4, 10, 20, 40, 200, 400, and 1 000 $ng \cdot mg^{-1}$). Methanol was added to the mixture ($V_{mixture}/V_{methanol}$ = 1/4) in order to precipitate protein, vortexed for approximately 2 min, and left standing for 10 min. The precipitate was removed by centrifugation at 14000 $r \cdot min^{-1}$ for 15 min. The supernatant (100 μL) was transferred into disposable glass auto-sampler vials. Finally, the sample (10 μL) was injected into the LC/MS/MS system for analysis. The lowest concentration on the calibration curve was set as the low limit of quantitation (LLOQ).

Precision and accuracy

Plasma control samples were spiked with three different concentrations (1000, 200, and 2 $ng \cdot mg^{-1}$) of the mixed standard solution. Five samples were prepared for each concentration in parallel, and determined five times on the same day for the intra-day, and once every day for three days consecutively for the inter-day accuracy test. The deviation of the mean test results (concentration) from the true value served as the measure of accuracy, and the coefficient of variation (CV, %) served as the measure of precision.

Stability

Three concentrations (1000, 200, and 2 $ng \cdot mg^{-1}$) of the plasma control samples spiked with the mixed standard solution were determined immediately (0 h), then set aside at room temperature (20 °C) for 3 h, 10 h, or frozen/thawed, three times within three days. The concentrations of the six standard components in these samples were determined and compared with the added amount, respectively (n = 5).

Recovery and matrix effects

Extraction recoveries and matrix effects were examined by comparing the peak areas of the different sample sets. In set 1, three concentrations of the mixed standard solution were dissolved in methanol. In set 2, three concentrations of the mixed standard solution were added to rat plasma blanks already subjected to methanol precipitation. In set 3, three concentrations of the mixed standard solution were spiked

with rat plasma blanks, and the resulting solutions then subjected to methanol precipitation (n = 5).

The recovery efficiency (RE) of sample was calculated as: RE = (mean peak area)$_{set3}$ / (mean peak area)$_{set2}$ × 100%. The absolute matrix effect (a-ME) was calculated as: a-ME = (mean peak area)$_{set2}$ / (mean peak area)$_{set1}$ × 100%.

Animals and experiments

Sprague-Dawley female rats (250 ± 20) g were provided by Release of Red Mountains Tianjin Laboratory Animal Technology Co. Ltd. (License No. SCXk2009-0001, Tianjin, China). The rats were housed with unlimited access to food and water except fasting for 12 h before the experiment. The animals were maintained on a 12 h light-dark cycle at ambient temperature and humidity each day. Animal experimental procedures were strictly conducted according to the Guidelines for the Care and Use of Laboratory Animals (National Research Council Of USA, 1996) and the related ethical regulations of this university (The animal care protocol approval number was TCM-LAEC 2013011).

Plasma pharmacokinetic studies in rats

Rats were randomly assigned to three groups (6 rats/group): the first group (G1) received a p.o. dose of *E. ulmoides* extract (500 mg·kg^{-1}); the second group (G2) received a p.o. dose of *D. asperoides* extract (1140 mg·kg^{-1}) and the third group (G3) received a mixed p.o. dose of *E. ulmoides* extract (500 mg·kg^{-1}) combined with *D. asperoides* extract (1140 mg·kg^{-1}). Each extract was milled with CMC-Na (0.4 %) prepared for oral administration. Serial blood samples (about 0.3 mL) were collected through the occuli chorioideae vein in heparinized tubes according to time schedules of 0, 5, 15, and 30 min, and then 1, 1.5, 2, 3.5, 5, 8, 10, and 24 h. The plasma was separated by centrifugation (14 000 r·min^{-1}, 15 min), and stored at −70 °C until analysis.

Statistical analysis

The values obtained for the pharmacokinetic parameters assessed among the three groups are expressed as $\bar{x} \pm s$. At the same time, the differences between the single administration *D. asperoides* extract alone (G1) or *E. ulmoides* extract alone (G2), and the co-administration of mixture of combined extracts (G3) were evaluated using the t-test. Values of $P < 0.05$ were considered statistically significant.

Results and Discussion

Method validation

Calibration curves and lower limit of quantitation

The calibration curves for the six compounds in rat plasma were linear in the range of 1–1 000 ng·mL^{-1}. The LLOQ values of AU, GP, GPA, PDG, SLG, and LG were 1 ng·mL^{-1}, and their accuracies 113.82%, 97.18%, 105.59%, 103.55%, 103.94%, and 105.78% (n = 5), respectively. The linear regression equations for the calibration curves of each ingredient were as follows:

AU: $y = 25.998\,0\,x + 28.062\,0$ ($r^2 > 0.993\,2$)

GP: $y = 81.326\,7\,x + 422.838\,0$ ($r^2 > 0.993\,5$)

GPA: $y = 11.690\,5\,x + 31.633\,9$ ($r^2 > 0.994\,1$)

PDG: $y = 81.712\,4\,x + 946.452\,2$ ($r^2 > 0.994\,3$)

SLG: $y = 13.211\,1\,x + 129.910\,9$ ($r^2 > 0.993\,1$)

LG: $y = 89.266\,6\,x + 328.629\,1$ ($r^2 > 0.992\,7$)

At the same time, using this analytical method, better separation was obtained for all of the six compounds, and in the blank sample there was no endogenous interference. The retention times were 3.039 (*RSD* = 0.07%), 6.128 (*RSD* = 0.04%), 5.771 (*RSD* = 0.08%), 6.040 (*RSD* = 0.04%), 6.186 (*RSD* = 0.01%), and 6.052 (*RSD* = 0.03%) min for the six compounds AU, GP, GPA, PDG, SLG, and LG, respectively (n = 6) (Fig. 2).

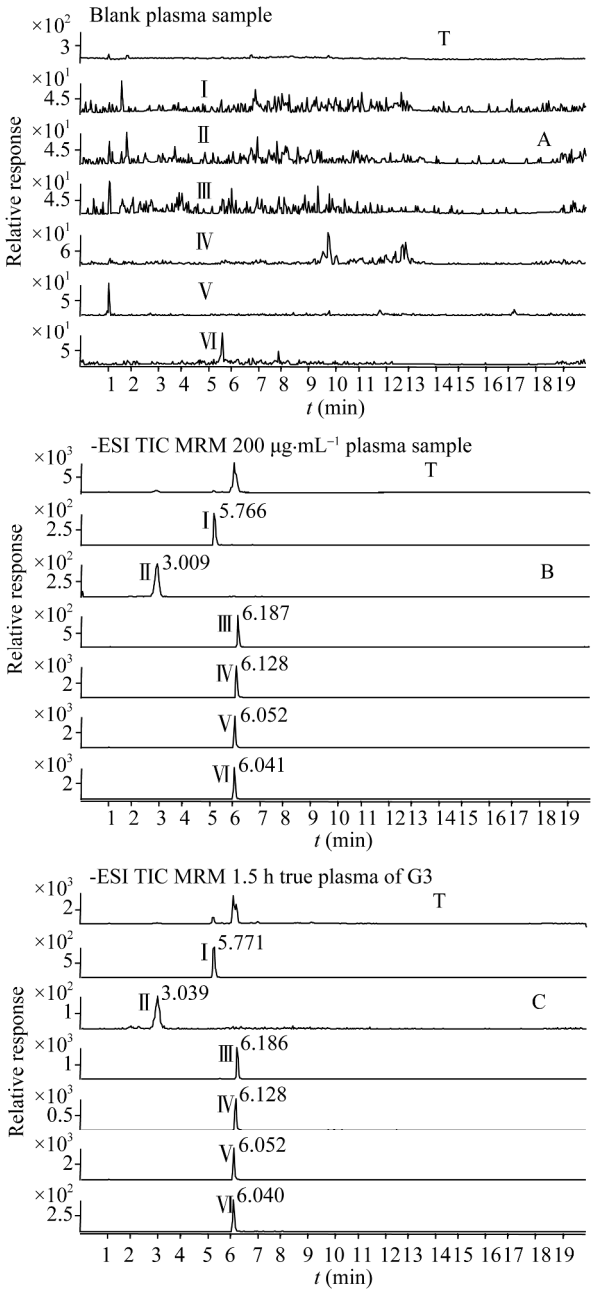

Fig. 2 Plots A, B, and C represent the blank plasma, 200 ng·mL^{-1} plasma sample, and 1.5 h true plasma of G3, respectively; I, II, III, IV, V, and VI show the MRM mass spectra of GPA, AU, SLG, GP, LG, and PDG, respectively. T represents

the total separation of the six compounds

Precision and accuracy

To assess the accuracy and precision of the developed analytical method, plasma samples containing mixed standard solutions at three concentrations (1 000, 200, and 2 ng·mL^{-1}) were analyzed using the method described above, and the results are shown in Table 1 (n = 5). Whether intra-day or inter-day, all compound precisions were no more than 9.70 %, and accuracies were within 85.11%–106.32%. These results indicated that this method had good precision and accuracy for each of the six compounds.

Stability

The stability studies revealed no significant degradation for any of the compounds, except for SLG, which was stable at ambient temperature (20 °C) and in one or two frozen/thawed cycles, but after three frozen/thawed cycles it had undergone significant degradation at low concentration (2 ng·mL^{-1}), and was decreased more than 50 %. The results are shown in table 2 (n = 3).

Table 1 Intra-day and inter-day stability tests for the six compounds in rat plasma

Compound	Concentration (ng·mL^{-1})	Intra-day (n = 5)		Inter-day (n = 3)	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
AU	2	85.11	3.01	96.01	4.34
	200	102.77	1.49	103.11	4.88
	1 000	97.15	4.70	103.58	9.70
GP	2	95.65	4.50	93.11	7.52
	200	103.01	5.41	105.86	2.91
	1 000	102.23	1.71	106.32	6.81
GPA	2	100.33	5.21	95.88	4.45
	200	99.16	3.99	98.10	2.98
	1 000	96.04	5.24	100.49	6.42
PDG	2	97.82	5.74	99.26	4.89
	200	99.49	4.05	98.47	1.32
	1 000	97.93	4.50	97.37	2.50
SLG	2	103.33	9.01	96.43	7.31
	200	104.05	4.12	103.59	2.92
	1 000	100.07	1.74	99.63	1.36
LG	2	101.13	3.65	99.01	4.01
	200	102.52	5.28	103.33	0.81
	1 000	98.80	4.11	99.78	1.22

Table 2 Stability study of six compounds in rat plasma (n = 3)

Compound	Nominal (ng·mL^{-1})	1 h post-procedure		10 h post-procedure		3 cycles	
		Accuracy (%)	*RSD*	Accuracy (%)	*RSD*	Accuracy (%)	*RSD*
AU	2	95.01	0.08	98.54	0.04	87.54	0.06
	200	95.25	0.03	105.18	0.05	92.12	0.04
	1 000	99.07	0.02	100.75	0.06	98.04	0.01
GP	2	106.38	0.08	101.55	0.07	89.77	0.05
	200	97.29	0.05	103.89	0.03	93.75	0.03
	1 000	98.94	0.01	99.78	0.04	96.85	0.01
GPA	2	89.86	0.04	117.51	0.16	93.31	0.38
	200	95.79	0.04	100.42	0.04	93.80	0.01
	1 000	93.61	0.01	97.19	0.01	94.32	0.01
PDG	2	101.57	0.07	99.58	0.04	101.22	0.04
	200	94.37	0.03	97.06	0.03	92.98	0.01
	1 000	94.34	0.01	96.66	0.03	98.42	0.03
SLG	2	95.32	0.03	98.66	0.09	87.33*	0.05*
	200	96.72	0.02	107.08	0.04	93.98	0.02
	1 000	94.98	0.01	97.91	0.03	95.41	0.01
LG	2	99.81	0.03	100.31	0.04	85.36	0.05
	200	100.06	0.04	103.30	0.03	96.63	0.03
	1 000	97.77	0.01	96.97	0.02	99.62	0.02

* represents the results of two frozen/thawed cycles

Recovery and matrix effect

At the three concentrations (1 000, 200, and 2 ng·mL^{-1}), the extraction recoveries for the six compounds were within 45.33%–77.59%, and the results of the investigation for matrix effect indicated that there was no significant suppression or enhancement effect. These results indicated that this sample handling was suitable for analyzing these compounds (Table 3).

Table 3 Matrix effects and recoveries of six compounds in rat plasma precipitated with MeOH (n = 5)

Compound	Nominal (ng·mL^{-1})	Recovery (%)	Matrix effect (%)
AU	2	50.12	1.40
	200	67.09	1.10
	1 000	70.23	1.00
GP	2	56.12	1.33
	200	66.01	1.15
	1 000	73.97	1.07
GPA	2	51.73	1.42
	200	64.57	1.14
	1 000	72.81	1.03
PDG	2	63.14	1.66
	200	65.75	1.15
	1 000	66.88	1.09
SLG	2	45.33	1.55
	200	64.60	1.10
	1 000	72.70	1.02
LG	2	60.77	1.61
	200	59.95	1.14
	1 000	77.59	1.06

Plasma pharmacokinetic studies

Pharmacokinetic parameters were obtained using DAS 1.0 pharmacokinetic software with the compartmental method (Table 4). For all the three groups, each of the analyzed compounds behaved as an open one-compartment model after intragastric administration, and their concentration–time profiles are shown in Fig. 3.

Although all the analyzed compounds in the three groups were present in the same dosage, following compatibility herb pair use, the pharmacokinetic properties of some of the compounds were changed. Comparing the pharmacokinetics of the analyzed compounds in the single extract administration group (G1, G2) with their pharmacokinetics in the combined extract administration group (G3), the values of t_{max} for AU and GP increased markedly ($P < 0.05$=, the values of c_{max} for AU and GP decreased markedly ($P < 0.05$=, while the pharmacokinetic parameters for all of the six compounds were not significantly different ($P > 0.05$). Compared with the study of Wang *et al* (2012), it was found in both studies that after administering only *E. ulmoides* extract, that the distribution of PDG followed the one compartment model, and did not markedly assume the bimodal model. But after administering the combined extract in this study, the distribution of PDG assumed the bimodal model. This indicated that the compatibility of the two plants might affect the pharmacokinetic properties (absorption, bile extraction and/or intestine reabsorption) of PDG, which might lead to the enterohepatic circulation of PDG. The specific mechanism of interaction requires further study.

Table 4 Main pharmacokinetic parameters of the six compounds in rat plasma following oral administration in three groups, expressed as $\bar{x} \pm s$ (n = 6)

Compound	Method	$AUC_{0\to24}$(ng·mL^{-1}·h)	$AUC_{0\to\infty}$(ng·mL^{-1}·h)	t_{max} (h)	c_{max} (ng·mL^{-1})	$t_{1/2}$ (h)
AU	G2	116.18 ± 49.39	149.37 ± 93.21	0.88 ± 0.31	80.09 ± 23.96	2.68 ± 1.17
	G3	127.14 ± 29.81	133.69 ± 31.12	2.50 ± 1.10	36.93 ± 17.45	2.26 ± 2.12
GP	G2	147.13 ± 74.24	147.41 ± 74.50	0.96 ± 0.60	113.44 ± 63.67	2.13 ± 0.79
	G3	132.56 ± 62.01	137.77 ± 60.66	1.92 ± 0.86	46.69 ± 23.43	1.50 ± 0.81
GPA	G2	2874.66 ± 1861.48	2875.16 ± 1861.80	2.08 ± 0.74	1020.42 ± 720.75	1.58 ± 0.37
	G3	1764.86 ± 1874.28	1770.03 ± 1878.75	2.58 ± 1.02	404.61 ± 429.47	2.73 ± 1.77
PDG	G2	48.73 ± 36.57	48.74 ± 36.58	1.75 ± 0.35	25.90 ± 20.90	Bimodal
	G3	23.27 ± 10.08	24.62 ± 9.66	0.75 ± 0.88	11.48 ± 0.94	Bimodal
SLG	G1	2889.25 ± 2209.66	2903.69 ± 2211.62	2.58 ± 1.07	733.83 ± 433.85	2.12 ± 0.93
	G3	3907.59 ± 2329.07	3957.13 ± 2313.64	2.83 ± 1.03	631.48 ± 274.06	3.40 ± 1.76
LG	G1	539.61 ± 386.53	544.01 ± 392.50	1.92 ± 0.92	156.65 ± 108.70	1.26 ± 0.43
	G3	569.39 ± 196.00	592.09 ± 207.05	1.94 ± 0.89	153.60 ± 49.38	1.36 ± 0.27

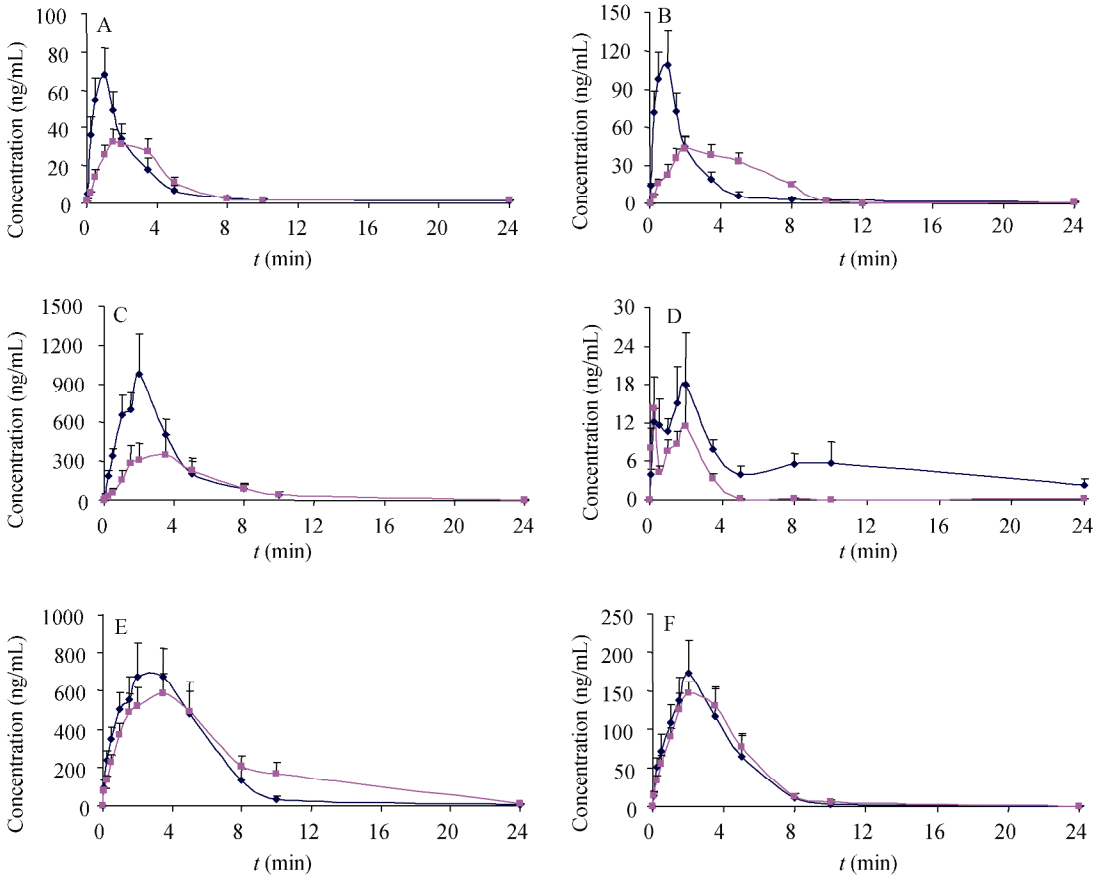

Fig. 3 The concentration–time profiles of the analyzed compounds following oral administration of the extract in rat plasma (*n* = 6). The blue line represents the concentration–time profiles following oral administration of *E. ulmoides* extract or *D. asperoides* extract only (G1, G2), the red line represents concentration–time profiles following oral administration of their mixture combined extracts (G3). A, B, C, D, E, and F represent AU, GP, GPA, PDG, SLG, and LG, respectively

Conclusion

A sensitive LC/MS/MS analytical method was developed and applied in the comparative pharmacokinetic studies of six active compounds in the plasma of rats dosed with the bark extract of *E. ulmoides* co-administered with *D. asperoides* root extract. With the results obtained from this study, it was found thar the t_{max} for AU and GP increased markedly ($P < 0.05$) and the c_{max} for AU and GP decreased markedly ($P < 0.05$) after administration of the combined extract (G3), indicating that the combination of plant medicines could result in a modification of the pharmacokinetics of the active ingredients contained in the formula. This conclusion might be useful for the further study of the mechanism of action of DuZhong Wan, in particular, and the mechanisms of action of TCMs in general.

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