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Comparative pharmacodynamic, pharmacokinetic and tissue distribution of Dahuang-Gancao decoction in normal and experimental constipation mice

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[ABSTRACT] Dahuang-Gancao decoction (DGD) is a classical formula, which is commonly used for reliving constipation in Chinese clinic. The aim of this study was to investigate the pharmacodynamic, pharmacokinetic and tissue distribution alternations of DGD in normal and constipation mice. DGD exhibited stronger purgative effect in constipation mice by the increased fecal excretion and reduced first defection time compared with normal mice. The C_{max}, $AUC_{0\text{-}t}$ and $MRT_{0\text{-}t}$ of rhein, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, and glycyrrhizic acid as main bio-active components in DGD were markedly increased in constipation mice. The tissue distribution of the analytes in constipation mice were higher than those in normal mice with rhein > rhein-8-*O*-*β*-D-glucoside > aloe-emodin > glycyrrhizic acid > emodin in liver, and glycyrrhizic acid > rhein-8-*O*-*β*-D-glucoside > liquitin > sennoside A > rhein > aloe-emodin > emodin in colon. The kidney concentrations of the analytes showed a descending order of rhein > rhein-8-*O*-*β*-D-glucoside > sennoside A > glycyrrhizic acid > aloe-emodin > emodin, most of them were higher while rhein was lower in constipation mice than normal mice. The higher exposure of the anthraquinones in plasma, liver and colon may result in the stronger purgative effect in the constipation mice than normal mice. Rhein is mainly excreted through the kidney, the decreased level of rhein in constipation mice may explain the alleviated side effects. Accumulation of glycyrrhizic acid in colon may related with the moderate property of licorice. These results provided the experimental basis for understanding the therapeutic effects and metabolite profile of DGD.

[KEY WORDS] Rhubarb; Licorice; Pharmacodynamic; Pharmacokinetic; Tissue distribution; Constipation

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Introduction

Constipation is a common gastrointestinal problem worldwide affects at least 8% of the individuals, occurring more often in females and older persons [1]. It is a disorder influenced by several factors including intake of dietary fiber, emotional influence, psychological morbidity, structural abnormality or systemic disease [2]. Treating constipation can be comprehensive, in many Asian countries, traditional Chinese medicine (TCM) such as *Rheum palmatum* (rhubarb), *Magnolia offcinalis*, *Rehmannia glutinosa*, *Cannabis sativa* is a popular choice [3]. Rhubarb was the most commonly used herb, however, it has been reported that the long-term use of rhubarb and the active ingredient causes drug resistance, whereby the therapeutic response is reduced, and adverse reactions including cathartic colon and melanosis coli, which may progress to intestinal pseudo-obstruction or colon cancer are induced [4-5].

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Dahuang-Gancao decoction (DGD) has a similar pugative effect as rhubarb but a lower incidence of side effects [6]. It is a classical formula recorded in Zhang Zhong-Jing's *Synopsis of the Golden Chamber*, which is composed of two herbs, rhubarb (Dahuang) and licorice (Gancao), that can be used for the treatment of constipation, excess heat stagnation of gastrointestinal tract and related symptoms [7]. The purgative effect of DGD is thought to be induced by anthraxquinones, the main components of rhubarb [8]. However, its purgative property is extremely strong and easy to cause adverse reactions. Thereby, licorice is combined with it to moderate the medicinal property and eliminate the side effect, which contains saponins and flavonoids as the major ingredients.

TCM containing several herbs and various ingredients may influence the *in vivo* behavior of each other, attributing additive, subtractive, synergistic, or antagonistic effects [9]. Multiple components detected simultaneously in TCM should be used to evaluate the integral pharmacokinetic profiles of TCM instead of the single component. There have been report about the pharmacokinetic of DGD, unfortunately, it only focused on the plasma concentration of rhein in rhubarb altered by licorice, and the result showed that combination of licorice can reduce the concentration of rhein [10], little attention has been paid to the changes of other ingredients in rhubarb and licorice. In addition, pharmacokinetic profiles change in different physiological states and disease progressions. More important information could be obtained through comparing the pharmacokinetics in different conditions [11]. However, little is known about the pharmacokinetics of DGD in in normal and constipation animals.

The objective of this study was to compare the pharmacokinetic and tissue distribution properties of main bio-active ingredients in DGD between the normal and constipation mice. Simultaneously, the effect of DGD in normal and constipation mice was investigated. Thereby, the *in vivo* behavior as well as pharmacological effects of DGD were evaluated to provide compelling evidence for the rational application of DGD for constipation.

Materials and Methods

Reagents and plant materials

The reference standards of rhein, emodin, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, glycyrrhizic acid, liquiritin, and chloramphenicol (internal standard, IS) (purity > 98%) were obtained from Chengdu Ruifensi Biological Technology Co., Ltd. (Chengdu, China). The chemical structures are shown in Fig. 1. Loperamide hydrochloride (LH) was purchased from Xi'an Yangsen Pharmaceutical Co., Ltd. (Xi'an, China). Acetonitrile and methanol were of HPLC grade and purchased from Merck (Darmstadt, Germany). Deionized water was purified by a Milli-Q Ultrapure water system (Bedford, USA).

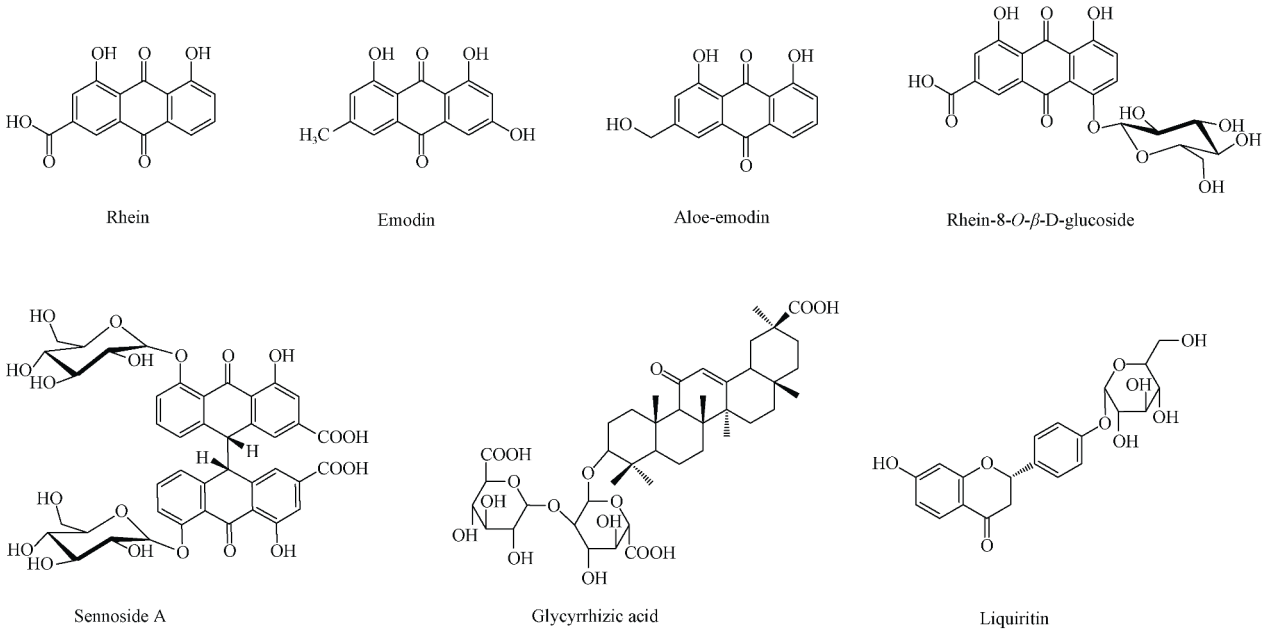

Fig. 1 Chemical structures of rhein, emodin, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, glycyrrhizic acid, and liquiritin

Rhubarb, the dried radix and rhizoma of *Rheum tanguticum* Maxim Balf., were collected from Gannan, Gansu province, China. Licorice, the dried radix and rhizoma of *Glycyrrhiza uralensis* Fisch., were collected from Linwu city, Ninxia province, China. The plant materials were identified by Prof. YAN Hui of Nanjing University of Chinese Medicine. The voucher specimen (Nos. 160326 and 151108) were deposited at the Herbarium in Jiangsu Key Laboratory for High Technology Research of TCM Formulae. DGD was prepared as follows: rhubarb and licorice were floured into power which can pass through 40 mesh sieve, and weighed according to the ratio of 4 : 1. The Chinese Pharmacopoeia mentioned the

usage of rhubarb as "The decoction time should be short for purgative effect". So licorice was refluxed with 8-fold water for 60 min, and 8-fold water was added to immerse rhubarb for 30 min, then the two herbs and extracts were mixed together and refluxed for 10 min. The extract was filtered immediately through 3 layers of gauze. After refluxing for another 2 times of 10 min with 6-fold water, the three filtrates were combined and rotary evaporated to the concentration of 2 g·mL^{-1}. The contents of the main bio-active compounds in DGD were measured quantitatively using UPLC-TQ/MS with the below LC conditions, chromatographic separations were achieved on a Thermo Scientific Hypersil Gold C_{18} column (100 mm × 2.1 mm, 1.9 μm) at 35 ºC, the flow rate was 0.40 mL·min^{-1}, and the injection volume was 5 μL. The contents of rhein, emodin, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, glycyrrhizic acid, and liquiritin were 45.450, 13.067, 36.149, 108.471, 54.693, 45.019, and 12.505 mg·mL^{-1}, respectively.

Experimental animals

Male ICR mice weighing 18−22 g were supplied by Nanjing Qinglongshan Laboratory Animal Co., Ltd., China. The mice were housed under controlled temperature (25 ± 1 °C), relative humidity (40%−70%), and a 12-h light/dark cycle for one week before use and fed with food and water *ad libitum*. All the procedures were in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Induction of constipation in mice

One hundred and eight mice were randomly divided into nine model groups (M1−M9, n = 6) and nine control groups (C1−C9, n = 6), and the experimental constipation was induced by oral administration of LH according to a protocol reported previously [12]. In brief, the model groups (n = 54) received 6 mg·kg^{-1} LH twice a day for 21 days, while the control groups (n = 54) received equivalent volumes of vehicle for 21 days. On the seventh day, the defecation characteristics of both groups were observed. Each mouse was given 0.2 mL red ink by gavage and placed in metabolic cage. The first defecation time was recorded and fecal samples were collected within 24 h. The numbers of red feces in 0−6 h were accounted, the wet and dry weight of feces in 0−12 h and 12−24 h were measured, and the water content of the fecal samples were calculated. From the eighth day on, each group were given DGD orally once a day for 14 days. On the twentieth day, the defecation characteristics of both groups were observed using the above method.

Pharmacokinetic and tissue distribution study

Plasma and tissue sample collection: Totally one hundred and fourteen mice were used, six mice without any treatment were used for blank plasma and tissues collection, and one hundred and eight mice described above were used for the follow-up experiment. On the last day, blood samples (500μL) were drawn from the oculi chorioideae vein at 5, 15, 30, 60, 120, 240, 360, 720, 1440 min after dosing, and placed into heparinized tubes and centrifuged at 3 000 r·min^{-1} for 10 min to obtain plasma. Mice were sacrificed and tissues including liver, kidney and colon were collected at 30, 60, 120, 360, 720, 1440 min, each time-point was based on samples from six different mice. Tissue samples were rinsed with physiological saline to remove the blood or content, blotted on filter paper, weighed rapidly, and then stored at –80 °C until analysis.

Plasma and tissue sample preparation

Plasma sample (200 μL) was mixed with 200 μL IS solution (10 ng·mL^{-1}) and 400 μL methanol. The mixture was vortexed for 1 min and centrifuged at 13 000 r·min^{-1} for 10 min. The supernatant was blown to dryness with nitrogen at 37 °C. Then the residue was re-constituted in 200 μL 80 % methanol for determination. Tissue sample was thawed and then homogenized in ice-cold physiological saline (W/V = 1/3). 500 μL of tissue homogenate was mixed with 200 μL IS solution (10 ng·mL^{-1}) and 1000 μL methanol. The mixture was vortexed for 1 min and then centrifuged at 13, 000 rpm for 10 min. The supernatant was evaporated and the residue was dissolved in 200 μL 80 % methanol for determination.

Liquid chromatography and mass spectrometry conditions

Chromatographic analysis was performed on a Waters Acquity UPLC system (Waters Corp., USA). A Thermo Hypersil Gold C_{18} column (100 mm × 2.1 mm, 1.9 μm) was employed and the column temperature was maintained at 35 ºC. The mobile phase was composed of A (0.1% formic acid) and B (acetonitrile) using a gradient elution as follow: 0−2 min, 5% B; 2−4 min, 5%−35% B; 4−8 min, 35%−95% B; 8−13 min, 95% B; 13−14 min, 95%−5% B. The flow rate was set at 0.40 mL·min^{-1} and the injection volume was 2 μL. Mass spectrometry detection was performed using a Xevo Triple Quadrupole MS (Waters Corp., USA) equipped with an ESI source. The parameters were set as follows: capillary voltage 3.0 kV, source temperature 150 ºC, desolvation temperature 550 ºC, cone gas flow 30 L·h^{-1}, desolvation gas flow 1000 L·h^{-1}. Quantification was obtained using the multiple reaction monitoring (MRM) acquisition in negative mode by monitoring the precursor ion to product ion transitions as listed in Table 1.

Table 1 Ion pairs for MRM scanning and ion spray source conditions

Analytes	t_R/min	Ion pair (m/z)	Cone voltages (V)	Collision energy (eV)
rhein	11.27	283→183	20	14
emodin	12.56	269→225	32	22
aloe-emodin	11.10	269→239	18	38
rhein-8-*O*-*β*-D-glucoside	5.86	445→283	26	22
sennoside A	5.48	861→224	30	22
glycyrrhizic acid	8.44	821→351	54	28
liquiritin	6.44	417→255	26	16
IS	7.82	321→152	30	16

Method Validation

The analytical method validation was performed according to the Food and Drug Administration guidance for bioanalytical method validation.

Specificity: The specificity of the method was evaluated by analyzing the chromatograms of blank matrix samples, blank matrix samples spiked with the mixed standards, and matrix samples after oral administration.

Calibration curve and lower limits of quantification (LLOQ): Calibration curve was prepared by spiking pooled blank matrix with an appropriate amount of mixed standards. Calibration curve was calculated using weighted linear regression by plotting peak area ratio of each analyte to IS versus concentrations of the analytes. LLOQ was defined as the lowest concentration in the calibration curve that can be quantitatively measured with a signal-to-noise ratio (S/N) at 10, precision lower than 20% and accuracy of 80%−120%.

Precision and accuracy: Quality control (QC) samples were prepared by individually spiking blank matrix samples with working solution at three concentration levels (high, middle and low). The intra-day and inter-day precision and accuracy were determined by analyzing six replicates of QC samples on three validation days. The precision was defined as the relative standard deviation (RSD). The accuracy was expressed as the relative error (RE) by calculating the percentage difference between the measured and spiked concentration over that of the spiked value.

Extraction recovery and matrix effect: The recovery and matrix effect of the analytes was conducted at three QC levels. The extraction recovery was determined by comparing the response ratio of extracted samples with extracted blank matrix spiked with corresponding concentrations. The matrix effect was evaluated by calculating the peak areas of analytes in extracted samples of blank matrix spiked with the analytes at corresponding concentration.

Stability: The stability of the analytes was assessed by analyzing six replicates of QC samples at three concentrations under different conditions. The following conditions were used: samples kept at room temperature for 12 h, samples stored at 4 ºC for 24 h, samples stored at –80 ºC for 20 days, and samples subjected to three freeze-thaw cycles.

Data Analysis

Values were expressed as mean ± SD. Pharmacokinetic parameters, including the maximum concentration (C_{max}), time to reach the maximum concentration (T_{max}), mean residence time from 0 to time ($MRT_{0\text{-}t}$), and the area under the blood concentration-time curve from 0 to time ($AUC_{0\text{-}t}$), the area under curve from 0 to infinity ($AUC_{0\text{-}\infty}$) were analyzed by a non-compartmental method using the DAS 2.0 pharmacokinetic program (Chinese Pharmacology Society).

Results

Characterization of the model and pharmacodynamic study

The defecation characteristics of both groups were observed after model establish on the seventh day and drug administration on the twentieth day. The results showed that the first defection time increased significantly, the numbers of red feces excreted within 6 hours and the amount of feces excreted in 0−12 h and 12−24 h decreased significantly in the model groups (M1−M9 on the 7th day) compared to the control groups (C1−C9 on the 7th day) (Table 2), which means that the constipation model was successfully replicated. After

Table 2 Effect of loperamide hydrochloride on defecation characteristics in normal and constipation mice on the seventh day (n = 6)

Group	The first defection t_R/min	The numbers of red feces in 0−6 h	The weight of feces in 0−12 h·g^{-1}	The weight of feces in 12−24 h·g^{-1}	The water content of feces in 0−24 h/%
C1-d7	269.5 ± 42.0	29.8 ± 5.2	1.31 ± 0.14	2.49 ± 0.54	0.49 ± 0.04
C2-d7	289.6 ± 31.5	30.5 ± 2.8	1.49 ± 0.22	2.68 ± 0.45	0.48 ± 0.03
C3-d7	277.6 ± 41.7	31.2 ± 1.9	1.37 ± 0.18	2.98 ± 0.33	0.52 ± 0.04
C4-d7	207.2 ± 32.1	28.7 ± 2.3	0.96 ± 0.14	2.31 ± 0.17	0.51 ± 0.04
C5-d7	239.8 ± 45.1	34.0 ± 2.1	1.78 ± 0.21	2.45 ± 0.31	0.50 ± 0.03
C6-d7	310.7 ± 29.6	28.9 ± 1.5	1.29 ± 0.18	2.19 ± 0.21	0.49 ± 0.02
C7-d7	355.9 ± 49.1	30.2 ± 3.7	1.57 ± 0.11	2.58 ± 0.33	0.51 ± 0.05
C8-d7	279.4 ± 31.2	30.1 ± 2.6	1.49 ± 0.31	2.11 ± 0.27	0.49 ± 0.03
C9-d7	301.5 ± 41.5	29.5 ± 1.6	1.41 ± 0.22	2.54 ± 0.42	0.52 ± 0.04
M1-d7	389.3 ± 42.7**	20.8 ± 2.4**	0.53 ± 0.11**	1.41 ± 0.16**	0.53 ± 0.05
M2-d7	359.6 ± 47.3**	19.9 ± 3.6**	0.57 ± 0.09**	1.43 ± 0.15**	0.50 ± 0.05
M3-d7	376.2 ± 31.6**	19.9 ± 3.6**	0.57 ± 0.09**	1.69 ± 0.28**	0.51 ± 0.03
M4-d7	379.0 ± 43.9**	19.7 ± 3.5**	0.56 ± 0.08**	1.29 ± 0.17**	0.47 ± 0.06
M5-d7	368.1 ± 44.1**	20.15 ± 3.1**	0.57 ± 0.04**	1.34 ± 0.14**	0.50 ± 0.05
M6-d7	362.4 ± 36.7**	19.9 ± 2.3**	0.56 ± 0.07**	1.28 ± 0.24**	0.48 ± 0.05
M7-d7	369.6 ± 34.3*	19.4 ± 1.9**	0.59 ± 0.05**	1.29 ± 0.23**	0.49 ± 0.04
M8-d7	379.5 ± 40.2**	18.9 ± 2.3**	0.57 ± 0.08**	1.37 ± 0.09**	0.49 ± 0.03
M9-d7	378.8 ± 26.1**	18.5 ± 1.8**	0.56 ± 0.07**	1.35 ± 0.25**	0.50 ± 0.04

$^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the mice in control group

oral administration of DGD, the time of the first defection in model groups was still longer than that of the control groups, and the numbers of red feces excreted within 6 hours and the amount of feces excreted in 0−12 h and 12−24 h in model groups (M1−M9 on the 20th day) were still less than that of the control groups (C1−C9 on the 20th day) (Table 3). However, the first defection time decreased, the numbers of red feces excreted within 6 hours and the amount of feces excreted in 0−12 h and 12−24 h increased significantly in both control and model groups on the twentieth day compared to that of on the seventh day. It indicated that DGD has obvious purgative effect on both normal and constipation mice, and the purgative extent on normal animal and constipation model is different.

Table 3 Effect of Dahuang-Gancao decoction on defecation characteristics in normal and constipation mice on the twentieth day (n = 6)

Group	The first defection t_R/min	The numbers of red feces in 0−6 h	The weight of feces in 0−12 h·g^{-1}	The weight of feces in 12−24 h·g^{-1}	The water content of feces in 0−24 h/%
C1-d20	213.0 ± 27.5$^{\triangle}$	32.3 ± 4.1	1.64 ± 0.10$^{\triangle}$	2.90 ± 0.34	0.51 ± 0.05
C2-d20	218.4 ± 35.2$^{\triangle}$	33.5 ± 3.3	1.89 ± 0.19$^{\triangle}$	3.12 ± 0.27$^{\triangle}$	0.48 ± 0.04
C3-d20	199.8 ± 38.7$^{\triangle}$	35.6 ± 2.8$^{\triangle}$	1.85 ± 0.20$^{\triangle}$	3.31 ± 0.35$^{\triangle}$	0.52 ± 0.04
C4-d20	173.2 ± 21.1	38.1 ± 3.3$^{\triangle\triangle}$	1.57 ± 0.22$^{\triangle}$	2.93 ± 0.22$^{\triangle}$	0.50 ± 0.03
C5-d20	181.4 ± 35.2	36.7 ± 2.5$^{\triangle}$	1.78 ± 0.21$^{\triangle}$	2.85 ± 0.29$^{\triangle}$	0.49 ± 0.04
C6-d20	249.3 ± 45.3$^{\triangle}$	34.6 ± 2.7$^{\triangle\triangle}$	1.79 ± 0.18$^{\triangle}$	2.99 ± 0.16$^{\triangle}$	0.50 ± 0.02
C7-d20	256.7 ± 50.2$^{\triangle}$	33.0 ± 2.8	1.87 ± 0.13$^{\triangle}$	3.02 ± 0.33$^{\triangle}$	0.52 ± 0.05
C8-d20	218.1 ± 32.0$^{\triangle}$	35.7 ± 3.6$^{\triangle}$	1.57 ± 0.23$^{\triangle}$	3.15 ± 0.26$^{\triangle}$	0.49 ± 0.04
C9-d20	231.6 ± 45.6$^{\triangle}$	34.4 ± 2.2$^{\triangle}$	1.82 ± 0.16$^{\triangle}$	3.04 ± 0.30$^{\triangle}$	0.51 ± 0.04
M1-d20	279.5 ± 52.7$^{*\triangle}$	31.2 ± 3.1$^{*\triangle\triangle}$	1.33 ± 0.21$^{*\triangle\triangle}$	2.52 ± 0.32$^{\triangle\triangle}$	0.48 ± 0.05
M2-d20	258.5 ± 37.4$^{*\triangle\triangle}$	29.1 ± 4.6$^{*\triangle\triangle}$	1.45 ± 0.14$^{*\triangle\triangle}$	2.34 ± 0.21$^{*\triangle\triangle}$	0.51 ± 0.03
M3-d20	267.2 ± 29.6$^{**\triangle\triangle}$	34.1 ± 3.6$^{\triangle\triangle}$	1.36 ± 0.15$^{*\triangle\triangle}$	2.28 ± 0.34$^{*\triangle}$	0.52 ± 0.04
M4-d20	247.0 ± 34.8$^{**\triangle\triangle}$	36.7 ± 5.5$^{*\triangle\triangle}$	1.46 ± 0.18$^{*\triangle\triangle}$	2.83 ± 0.36$^{\triangle\triangle}$	0.48 ± 0.06
M5-d20	258.5 ± 51.2$^{**\triangle}$	35.5 ± 5.6$^{\triangle\triangle}$	1.27 ± 0.17$^{*\triangle\triangle}$	2.58 ± 0.29$^{\triangle\triangle}$	0.51 ± 0.04
M6-d20	252.9 ± 32.6$^{\triangle\triangle}$	32.8 ± 3.7$^{\triangle\triangle}$	1.35 ± 0.09$^{*\triangle\triangle}$	2.43 ± 0.31$^{*\triangle\triangle}$	0.48 ± 0.05
M7-d20	231.4 ± 43.2$^{\triangle\triangle}$	34.5 ± 2.8$^{\triangle\triangle}$	1.46 ± 0.20$^{*\triangle\triangle}$	2.35 ± 0.26$^{*\triangle\triangle}$	0.49 ± 0.04
M8-d20	225.3 ± 47.1$^{\triangle\triangle}$	34.6 ± 3.2$^{\triangle\triangle}$	1.49 ± 0.13$^{\triangle\triangle}$	2.62 ± 0.17$^{*\triangle\triangle}$	0.50 ± 0.03
M9-d20	268.4 ± 31.7$^{*\triangle\triangle}$	33.6 ± 3.8$^{*\triangle\triangle}$	1.57 ± 0.23$^{*\triangle\triangle}$	2.37 ± 0.35$^{*\triangle\triangle}$	0.49 ± 0.05

$^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the mice in control group; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ *vs* the mice before administration of Dahuang-Gancao Decoction

LC-MS/MS method validation

Specificity: Specificity was assessed by analysis of blank matrix samples (plasma or tissue homogenate) with and without spiking with mixed standards. Fig. 2 shows the representative LC-MS/MS chromatograms of blank plasma samples, blank plasma samples spiked with standards. There was no endogenous interference from biological matrix at eluting positions of each standard in the chromatogram.

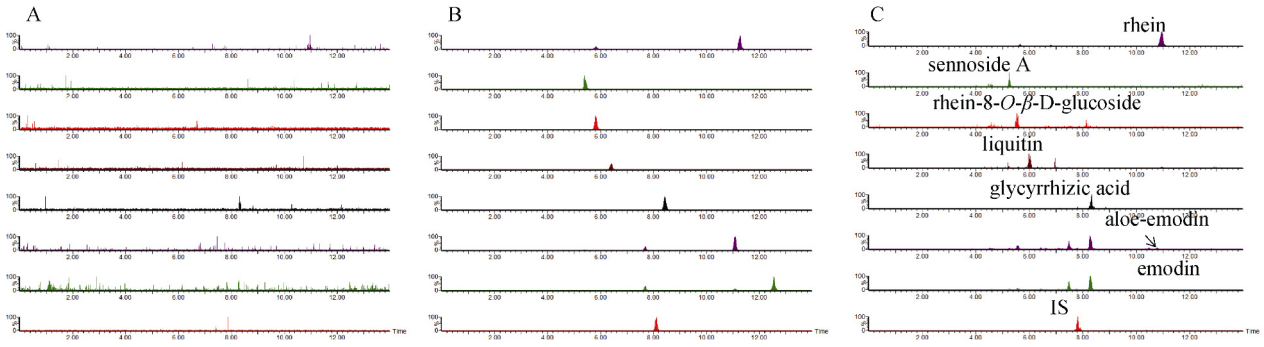

Fig. 2 The representative LC-MS/MS chromatograms of blank plasma sample, blank plasma sample spiked with standards and plasma sample after oral administration of DGD

Calibration curve and LLOQ: The calibration curves showed good linearity, all of the correlation coefficients were higher than 0.9983. The results showed that there was good correlation between the ratio of peak area and concentration for each compound within the linearity ranges. The typical equation of calibration curves, linearity ranges, and LLOQ for the seven analytes in mice plasma were shown in Table 4.

Precision and accuracy: The results showed that this LC-MS/MS method had satisfactory reproducibility with precision and accuracy data for intra-day and inter-day analyses less than 15% at three concentration levels. The precision

(RSD) was less than 9.7 % and accuracy (RE) was ranging from -6.5% to 12.2% in plasma (Table 5), precision (RSD) was less than 6.8%, 10.5%, 7.4% and accuracy (RE) was ranging from −3.1% to 9.7%, −7.1% to 10.5%, 2.5% to 8.6% in liver, kidney, and colon, respectively.

Extraction recovery and matrix effect: The extraction recoveries of analytes were analyzed at three concentration levels and all the values were in the range of 78.2%−110.4%, with 78.2%−91.3% in plasma (Table 5), 81.8%−95.2% in liver, 88.4%−110.4% in kidney, and 85.6%−97.9% in colon. The matrix effect of analytes was found to be within the acceptable range; all values were varied from 80.2%−98.7%, with 80.2%−92.8% in plasma (Table 5), 83.5%−96.1% in liver, 87.4%−98.7 % in kidney, and 81.5%−92.7 % in colon.

Table 4 Calibration curves, correlation coefficients, linear ranges, and LLOQ of the analytes in plasma

Analytes	Calibration curves	R^2	Linearity ranges (ng·mL^{-1})	LLOQ (ng·mL^{-1})
rhein	$Y = 0.0277X - 0.0102$	0.9983	9.306−18612	9.306
emodin	$Y = 0.0167X - 0.0983$	0.9995	2.265−4530	2.265
aloe-emodin	$Y = 0.0598X - 0.0641$	0.9994	9.425−9425	9.425
rhein-8-*O*-*β*-D-glucoside	$Y = 0.5057X + 0.311$	0.9993	28.86−5772	28.86
sennoside A	$Y = 0.3188X + 0.0388$	0.9997	27.00−5400	27.00
glycyrrhizic acid	$Y = 0.1979X - 0.0362$	0.9989	11.59−11590	11.59
liquiritin	$Y = 0.0969X - 0.0465$	0.9988	8.669−8669	8.669

Table 5 Precision, accuracy, recovery and matrix effect of the analytes in plasma

Analytes	C (ng·mL^{-1})	Intra-day		Inter-day		Recovery (mean ± SD, %)	Matrix effect (mean ± SD, %)
		Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)		
rhein	18.61	6.2	4.5	5.8	4.2	89.5 ± 2.7	80.2 ± 3.0
	186.1	5.3	2.3	7.4	9.2	86.3 ± 3.1	81.7 ± 5.3
	3722	7.2	5.8	3.9	2.7	90.2 ± 3.2	88.1 ± 2.8
emodin	4.530	4.1	5.1	9.7	-6.5	81.6 ± 6.8	89.3 ± 4.3
	45.30	3.9	–4.1	5.5	3.0	82.7 ± 4.4	92.8 ± 1.3
	226.5	6.5	3.8	3.2	-2.2	89.3 ± 4.9	84.8 ± 2.5
aloe-emodin	18.85	7.8	7.2	5.1	7.3	78.2 ± 5.3	81.5 ± 6.5
	188.5	3.2	4.5	5.6	6.4	81.7 ± 6.0	83.8 ± 6.9
	942.5	6.0	7.1	3.4	5.2	86.4 ± 3.2	80.9 ± 2.0
rhein-8-O-β-D-glucoside	57.72	3.1	-5.6	5.9	7.0	84.5 ± 8.5	83.7 ± 6.3
	577.2	5.8	4.5	4.5	4.7	90.3 ± 3.1	89.1 ± 2.8
	2886	2.3	7.0	6.8	3.5	85.9 ± 3.7	91.3 ± 3.3
sennoside A	27.00	7.7	12.2	2.9	4.2	79.4 ± 9.3	78.5 ± 4.2
	270.0	5.3	5.7	4.6	2.9	85.9 ± 6.4	82.5 ± 6.7
	540.0	4.8	3.7	3.1	-2.6	87.5 ± 4.5	81.4 ± 8.1
glycyrrhizic acid	23.18	3.2	-3.2	2.8	12.1	85.8 ± 2.7	82.9 ± 7.1
	231.8	2.9	4.3	2.1	5.8	91.3 ± 3.8	90.8 ± 5.7
	4636	1.6	2.1	3.7	6.4	87.5 ± 3.1	85.3 ± 2.4
liquiritin	17.34	2.8	5.0	2.7	5.2	89.6 ± 9.5	87.5 ± 1.5
	173.4	5.2	7.8	3.5	4.4	85.7 ± 2.6	89.6 ± 3.7
	867.0	5.4	3.3	4.3	4.8	83.8 ± 3.7	84.2 ± 4.1

Stability: Stability of the analytes during the sample storing and processing procedures was fully evaluated by analysis of QC samples. The results showed that all the values were within the range of 81.9%−103.2% in plasma (Table 6), 85.3%−99.1% in liver, 87.8%−107.4% in kidney, and 85.4% −102.7% in colon, which indicated that these analytes in mice plasma and tissues were all stable for one-month storage at –80 °C, 24 h in the auto-sampler (4 °C), 12 h at room temperature and three freeze-thaw cycles.

Pharmacokinetic study

Under the validated LC-MS/MS method, four anthraquinones, glycyrrhizic acid and liquiritin could be detected in control and conspitation mice plasma. The mean plasma concentration-time curves were shown in Fig. 3, and the main pharmacokinetic parameters were listed in Table 7. In general, the C_{max}, $AUC_{0\text{-}t}$, and $AUC_{0\text{-}\infty}$ values of rhein, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, and glycyrrhizic acid in model group were significantly higher compared with those

in control group. The T_{max}, $T_{1/2}$ values were similar between model group and control group, only $T_{1/2}$ of rhein-8-*O*-*β*-D-glucoside exhibited significant differences. In addition, $MRT_{0\text{-}t}$ of rhein, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, and glycyrrhizic acid were significantly prolonged in model group. These data indicate a higher systemic exposure of anthraquinones and glycyrrhizic acid in conspitation mice than normal mice.

Table 6 Stability tests of the analytes in plasma

Analytes	C (ng·mL^{-1})	Remaining (mean ± SD, %)			
		Short-term stability	Long-term stability	Freeze–thaw stability	Post-preparative stability
rhein	18.61	87.2 ± 6.4	80.5 ± 4.4	83.7 ± 5.9	93.3 ± 5.2
	186.1	94.1 ± 4.3	85.1 ± 5.1	92.9 ± 5.5	96.1 ± 3.7
	3722	97.5 ± 3.7	88.3 ± 4.9	98.2 ± 5.1	89.2 ± 6.3
emodin	4.530	94.2 ± 5.7	85.3 ± 4.2	94.2 ± 6.3	91.4 ± 3.8
	45.30	87.3 ± 5.2	98.1 ± 4.7	87.6 ± 3.2	94.8 ± 5.6
	226.5	92.1 ± 3.9	89.3 ± 6.4	91.3 ± 5.4	95.9 ± 3.1
aloe-emodin	18.85	87.5 ± 5.3	91.2 ± 4.6	92.1 ± 4.8	89.5 ± 5.9
	188.5	88.4 ± 2.5	92.5 ± 3.5	88.2 ± 6.7	92.1 ± 4.3
	942.5	89.5 ± 2.7	99.4 ± 4.2	94.9 ± 2.4	88.3 ± 5.8
rhein-8-*O*-*β*-D-glucoside	57.72	82.9 ± 7.3	84.8 ± 5.5	87.5 ± 5.2	85.6 ± 6.2
	577.2	90.5 ± 4.4	82.7 ± 1.1	89.8 ± 8.3	91.5 ± 5.5
	2886	88.2 ± 5.9	85.1 ± 4.4	91.7 ± 6.8	92.6 ± 3.7
sennoside A	27.00	94.4 ± 5.2	81.9 ± 7.0	88.9 ± 7.2	84.1 ± 4.2
	270.0	91.5 ± 2.8	85.7 ± 5.1	90.8 ± 3.1	96.2 ± 2.4
	540.0	93.2 ± 5.9	91.1 ± 4.2	94.9 ± 2.3	89.3 ± 5.4
glycyrrhizic acid	23.18	85.4 ± 2.9	93.5 ± 3.6	88.2 ± 3.7	92.1 ± 5.3
	231.8	98.2 ± 2.3	86.5 ± 3.4	92.2 ± 4.5	96.3 ± 1.6
	4636	92.1 ± 3.9	103.2 ± 6.4	93.5 ± 5.4	102.9 ± 3.8
liquiritin	17.34	95.2 ± 5.4	90.6 ± 4.1	90.8 ± 3.5	88.6 ± 5.5
	173.4	91.1 ± 3.7	92.4 ± 3.2	88.9 ± 5.2	89.3 ± 7.8
	867.0	87.4 ± 6.0	89.9 ± 5.3	85.8 ± 6.4	95.0 ± 4.2

Tissue distribution study

The distribution of rhein, emodin, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, glycyrrhizic acid, and liquiritin in liver, kidney, and colon of control and conspitation mice were shown in Figs. 4−6. Five analytes could be detected in liver, the concentration of rhein, emodin, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, and glycyrrhizic acid in model group were higher than those in control group. Six analytes could be detected in kidney, the concentration of rhein and emodin in model group were lower than those in control group, while the concentration of rhein-8-*O*-*β*-D-glucoside, sennoside A, and glycyrrhizic acid in model group were higher than those in control group. The maximum peak time of rhein, emodin and aloe-emodin in liver and kidney was about 30 min, while that of rhein-8-*O*-*β*-D-glucoside, sennoside A, and glycyrrhizic acid was about 60 min. However, these analytes could not be detected in liver and kidney beyond 1440 min, which suggested little accumulation in liver and kidney. Rhein, emodin, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, glycyrrhizic acid, and liquiritin could be detected in colon, and these concentrations in model group were higher than that in control group. The maximum peak time of those analytes in colon was about 360 min, which was much longer than that in liver and kidney. These data indicate a higher liver and colon exposure of anthraquinones and glycyrrhizic acid in conspitation mice than normal mice.

Discussion

As a well-known traditional Chinese formula, DGD is composed of rhubarb and licorice and has been used for the treatment of constipation for centuries. Rhubarb and licorice contains many components, of which anthraquinones are considered to be the most important chemical types exhibiting purgative effect in rhubarb, and saponins and flavonoids are considered to be the major chemical types in licorice playing the role of clearing away heat and relieving side effects. To compare the absorption extent of the major components, the dose normalized $AUC_{0\text{-}\infty}$ data was calculated. When normalized with the respective dose, the systemic exposure of each component showed a descending order of rhein > glycyrrhizic acid > liquiritin > sennoside A > rhein-8-*O*-*β*-D-glucoside > aloe-emodin. There are previous study reports that aloe-emodin, sennoside A, and rhein-8-*O*-*β*-D-glucoside could be biotransformed to rhein [13], which could partially explain their relatively lower systemic exposures. Compared with rhubarb, the purgative effect of DGD is moderate, the addition of licorice is of importance. As one of the most important biological ingredient of licorice, glycyrrhizic acid possess mutiple pharmacological effects such as analgesia, spasmolysis, anti-inflammatory, dotoxitificaiton [14-15]. The high exposure of glycyrrhizic acid in plasma may explain the moderate property of licorice.

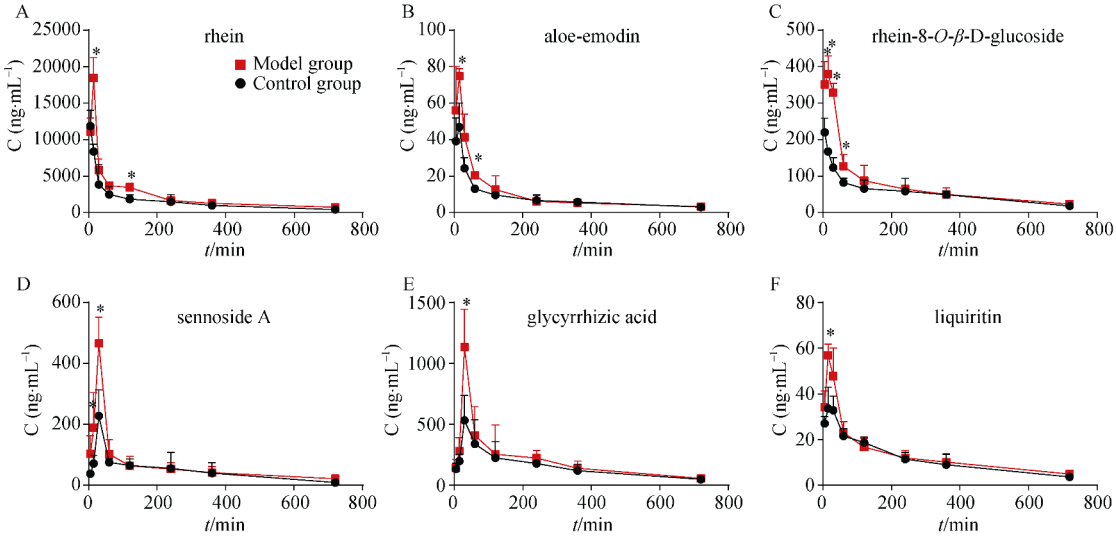

Fig. 3 Mean plasma concentration-time curves of rhein, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, glycyrrhizic acid and liquiritin in control and constipation mice after oral administration of DGD. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the control group

Table 7 Pharmacokinetic parameters of analytes in control and conspitation mice

Analytes	Group	C_{max} (ng·mL^{-1})	t_{max} /min	$t_{1/2}$ /min	$MRT_{0\text{-}t}$ /min	$AUC_{0\text{-}t}$ (ng·h·mL^{-1})	$AUC_{0\text{-}\infty}$ (ng·h·mL^{-1})
rhein	C	11866.85 ± 2692.71	5.00 ± 0.00	28.34 ± 3.65	44.44 ± 3.19	18157.34 ± 567.23	20678.55 ± 573.64
	M	18514.25 ± 3593.13**	13.33 ± 4.08*	30.24 ± 1.76	63.75 ± 4.28*	24379.26 ± 789.02**	28996.76 ± 773.52**
aloe-emodin	C	72.44 ± 28.38	5.00 ± 0.00	17.56 ± 2.55	73.23 ± 5.29	98.63 ± 13.53	112.45 ± 11.58
	M	106.58 ± 16.31**	5.00 ± 0.00	18.39 ± 3.78	96.74 ± 1.55*	123.54 ± 7.89**	137.66 ± 8.03**
rhein-8-*O*-*β*-D-glucoside	C	245.05 ± 68.15	5.00 ± 0.00	29.12 ± 4.01	73.23 ± 3.35	718.33 ± 22.05	774.27 ± 22.47
	M	442.61 ± 56.80**	15.00 ± 0.00	43.74 ± 3.11**	122.99 ± 7.33**	1062.50 ± 28.96**	1104.83 ± 27.99**
sennoside A	C	226.41 ± 45.27	30.00 ± 0.00	51.24 ± 2.23	75.15 ± 6.39	586.25 ± 15.65	603.27 ± 14.78
	M	496.00 ± 71.54**	30.00 ± 0.00	52.85 ± 3.57	153.52 ± 7.44**	821.46 ± 24.68**	885.54 ± 25.89**
glycyrrhizc acid	C	473.85 ± 52.37	30.00 ± 0.00	58.23 ± 4.89	80.64 ± 2.13	1933.59 ± 30.89	2068.42 ± 29.93
	M	736.0 ± 72.77**	30.00 ± 0.00	53.92 ± 2.06	116.23 ± 4.39*	2254.15 ± 48.92**	2407.39 ± 45.96**
liquiritin	C	41.04 ± 12.97	15.00 ± 0.00	70.24 ± 3.55	178.44 ± 3.81	141.49 ± 25.13	152.17 ± 23.76
	M	54.98 ± 7.86	15.00 ± 0.00	63.04 ± 2.54	180.17 ± 7.80	153.22 ± 23.66	169.56 ± 24.07

$^{*}P < 0.05$, $^{**}P < 0.01$*vs* the control group

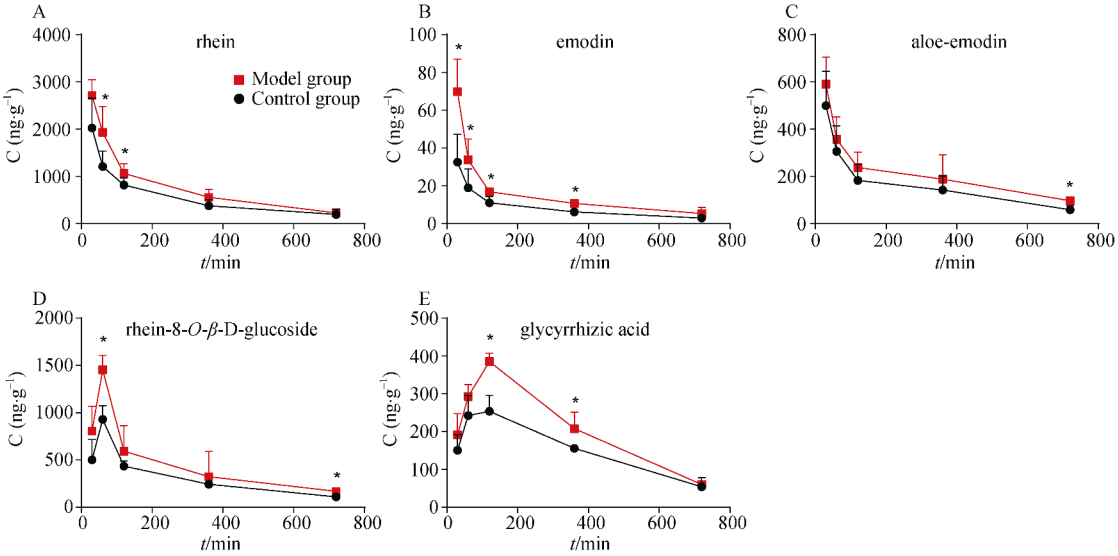

Fig. 4 Mean concentration-time curves of rhein, emodin, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, and glycyrrhizic acid in liver of control and constipation mice. $^{*}P < 0.05$ *vs* the control group

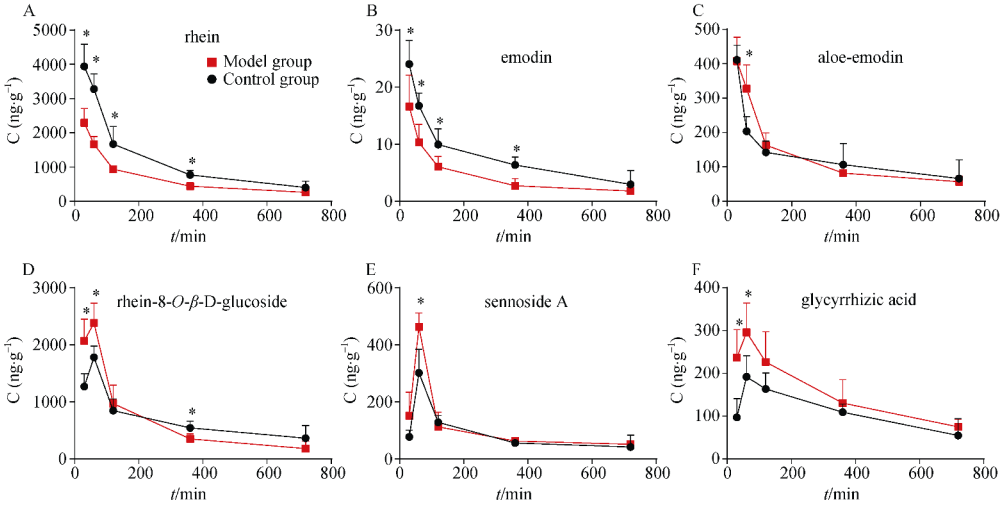

Fig. 5 Mean concentration-time curves of rhein, emodin, aloe-emodin, rhein-8-O-*β*-D-glucoside, sennoside A, and glycyrrhizic acid in kidney of control and constipation mice. $^{*}P < 0.05$ *vs* the control group

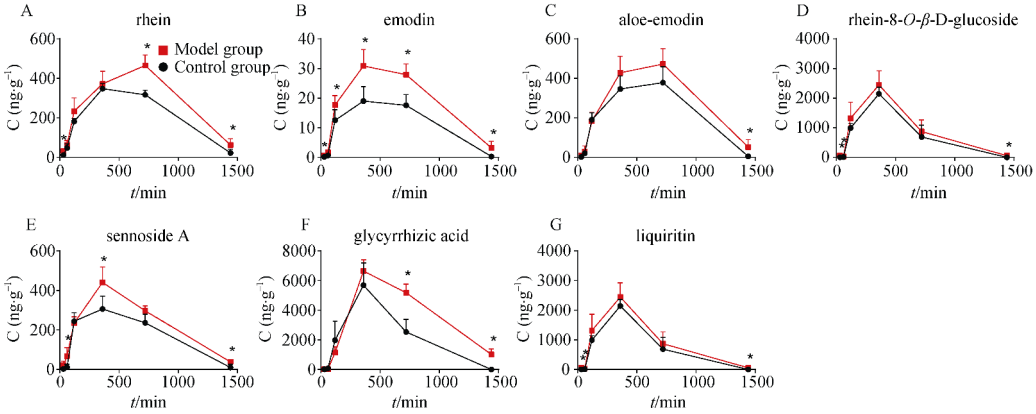

Fig. 6 Mean concentration-time curves of rhein, emodin, aloe-emodin, rhein-8-O-*β*-D-glucoside, sennoside A, glycyrrhizic acid in colon of control and constipation mice. $^{*}P < 0.05$ *vs* the control group

Pharmacokinetic study of traditional Chinese formula in normal animals are abundant, however, drug therapy is directed against disease, which makes the study in diseased animals important. There is still limited information on the pharmacokinetic profile of DGD in model animals, thereby the comparative pharmacodynamic, pharmacokinetic profile in normal and constipation animals was investigated here. LH induced constipation inhibits colonic peristalsis, and this inhibition extents fecal evacuation time and delays intestinal luminal transit [16]. DGD exhibited purgative effect on both normal and constipation mice by increasing the numbers, the weight of feces and reducing the first defection time, but the purgative extent on normal and constipation mice is different. In order to investigate whether pharmacokinetic profiling of DGD can be altered under constipation, the pharmacokinetic characteristics of key ingredients in DGD were measured. It was found that the important pharmacokinetic parameters of C_{max}, $AUC_{0\text{-}t}$ and $MRT_{0\text{-}t}$ were markedly different in the constipation mice. C_{max} of rhein, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, and glycyrrhizic acid was significantly increased by 1.6, 1.5, 1.8, 2.2, 1.6-fold and $AUC_{0\text{-}t}$ of those analytes was significantly increased by 1.3, 1.3, 1.5, 1.4, 1.2-fold respectively compared to normal group. This suggested that constipation increased the absorption of rhein, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, and glycyrrhizic acid and retained their excretion in the gastrointestinal system. It is well documented that LH induced delays in colonic transit is a spastic constipation due to the increase of colonic contractions in animals [17] and the inhibition on defection frequency in humans [18], thereby $MRT_{0\text{-}t}$ of rhein, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, and glycyrrhizic acid in model group was significantly prolonged by 1.4, 1.3, 1.7, 2.0, and 1.4-fold respectively due to the slow transit of intestinal motility induced by LH. Surprisingly, the change of absorption rate was not obvious, only T_{max} of rhein and $T_{1/2}$ of rhein-8-*O*-*β*-D-glucoside prolonged significantly, which may due to the biotransformation of rhein-8-*O*-*β*-D- glucoside to rhein.

To further understand the *in vivo* behavior of DGD, tissue distributions of key ingredients in liver, kidney and colon were studied. The liver concentrations of the analytes in constipation mice were higher than those in normal mice with rhein > rhein-8-*O*-*β*-D-glucoside > aloe-emodin > glycyr-

rhizic acid > emodin. Rhein is mainly metabolized in the liver, bile excretion and enterohepatic circulation of rhein and its metabolites in rats increase the concentration and time [19], which may cause the high concentration of rhein. The kidney concentrations of the analytes showed a descending order of rhein > rhein-8-*O*-*β*-D-glucoside > sennoside A > glycyrrhizic acid > aloe-emodin > emodin. Interestingly, the kidney concentrations of rhein in constipation mice were significantly lower than that in control mice. Rhein is mainly excreted through the kidney, and it has been recognized as a toxic component contributing to the kidney toxicity of rhubarb [20], the decreased level of rhein in constipation mice may explain the alleviated side effects. The colon concentrations of the analytes in constipation mice were higher than those in normal mice with glycyrrhizic acid > rhein-8-*O*-*β*-D-glucoside > liquitin > sennoside A > rhein > aloe-emodin > emodin. Constipation is a condition in which bowel movements are infrequent, irregular, or difficult [21], Licorice can prevent the severe contraction of colon caused by rhubarb and plays an important role in relieving abdominal pain at the beginning of diarrhea, which may closely be related with the high exposure of glycyrrhizic acid in colon.

Gut microbiota, known as a complex ecological community colonized in gastrointestinal tract, can interact with host factors to affect normal physiology and diseases. There was previous study about DGD and gut microbiota [6], the result showed that DGD administration could reduce the family Ruminococcaceae and increase the family Enterobacteriaceae, which suggested that the abundance of the family Enterobacteriaceae induced by DGD administration was associated with the purgative activity. However, the relationship between altered gut microbiota and changed pharmacokinetic behavior need further investigation.

Conclusion

An UPLC-MS/MS method for the quantification of key ingredients in DGD (rhein, emdin, aloe-emodin, rhein-8-*O*-*β*-D-glucoside, sennoside A, glycyrrhizic acid, and liquiritin) and its application to determine the comparative pharmacokinetic and tissue distribution in mice were developed and validated. The comparative pharmacodynamic, pharmacokinetic and tissue distribution of DGD in normal and constipation mice are reported here. The increased fecal excretion in model group compared to normal group indicated that the laxative degree induced by DGD varied from physiological conditions. The increased absorption extent in plasma, liver and colon in model group was determined, which may affect the contribution to the purgative effect, and maybe useful for the clinical prescription.

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