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# Sensitive and robust UPLC–MS/MS method to determine the gender-dependent pharmacokinetics in rats of emodin and its glucuronide

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ABSTRACT

In this study, a sensitive and robust ultraperformance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method was developed, validated, and applied to determine gender-dependent pharmacokinetics of total emodin (aglycone + glucuronide) in male and female Sprague–Dawley rats. The lower limit of quantification for emodin and emodin glucuronide in rat plasma was 39 and 78 ng/ml, with signal-to-noise ratio of ≥10. Precision and accuracy studies showed emodin and emodin glucuronide plasma concentrations well within the 10% range in all studies. Plasma recovery of emodin and emodin glucuronide was always above 86% for low (emodin: 39 ng/ml; glucuronide: 78 ng/ml), 92% for medium (625 ng/ml), and 97% for high (10 000 ng/ml) concentrations. Furthermore, emodin showed more than 95% plasma stability under short-term and long-term storage conditions, as well as after three freeze–thaw cycles in the experiments. The developed and validated analytical method was successfully applied to study the gender-dependent 10-fold higher oral bioavailability of total emodin in male than female rats. The oral bioavailability of emodin and emodin glucuronide was also measured separately and showed a statistically significant gender difference in oral bioavailability of emodin and emodin glucuronide in rats.



## 1. Introduction

Emodin (1, 3, 8-trihydroxy-6-methylanthraquinone) (Fig. 1) is one of the major active components of rhubarb (*Rheum officinale* B.), aloe (*Aloe barbadensis* M.), and leaf of senna (*Cassia angustifolia*). In the past decades, emodin has been extensively studied for its traditional pharmacological activities. However, recent studies have placed emodin back into the limelight with its anti-cancer activities against several types of cancer cells, with apoptosis as a possible mechanism of action [1,2]. Emodin also has an inhibitory effect on cancer cell migration [3] and invasion [4] in *in vitro* studies. Emodin has good prospects [5] and a promising future in anticancer treatment [6].

In our previous studies, emodin showed extremely fast metabolism in rat intestine and liver microsomes from five animal species, including mice, dogs, and humans [7]. Moreover, intestinal absorption and disposition of emodin were investigated in rats using an *in situ* intestinal perfusion model. After conducting *in vitro* and *in situ* studies, we confirmed that Phase II metabolism (glucuronidation) was the major route of emodin metabolism in rats. Although emodin absorption and disposition studies have been conducted *in vitro* and *in situ*, limited information is available regarding its oral bioavailability and in vivo disposition.

In the last decade, majority of research has been focused on pharmacokinetics of Da-Cheng-Qi decoction (DCQD), a Chinese medicine formulation composed of emodin and rhein. Gong et al. [8] showed gender-dependent (female > male) oral bioavailability of emodin after single dose oral administration of DCQD in rats. The authors suggested no food interaction in oral bioavailability of emodin during the study, although quantification of six different compounds in DCQD formulation suggested inter-variable plasma pharmacokinetic (PK) profiles in rats [9]. Similar to DCQD formulation PK studies, plasma concentrations of emodin were determined using various Chinese medicine formulae, such as San-Huang-Xie-Xin decoction [10], gan kang-granules [11], and Xie Xin decoction [12]. However, a critical point was missed in the literature search in that no absolute oral bioavailability study using orally administered unformulated and pure emodin has been conducted until now, with only two exceptions [13,14]. Emodin (4 mg/kg) oral administration showed very poor oral bioavailability in rabbits

*Abbreviations:* UPLC, ultra-performance liquid chromatography; IS, internal standard; DP, declustering potential; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential; AUC, area under the curve; DCQD, Da-Cheng-Qi decoction; QC, quality control; LLOQ, lower limit of quantification.

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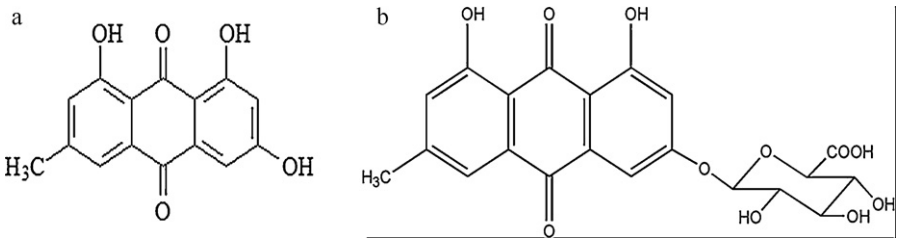


**Fig. 1.** The structures of (a) emodin and (b) emodin 3-*O*-β-D-glucuronide.

(<1%) [13] and rats (<3%) [14]. Furthermore, emodin glucuronide (Fig. 1) was accurately theorized as the predominant metabolite of emodin without a proper rat plasma emodin glucuronide profile by analyzing rat plasma samples using HPLC [13]. The lack of emodin glucuronide plasma PK profile might be due to limitations in the sensitivity of the HPLC instrument. To date, no information regarding the absolute oral bioavailability of emodin in rats is available. There is also lack of information regarding gender effect on absolute oral bioavailability of emodin in rats. Therefore, in this study, we determined the rat plasma PK profiles of emodin and its conjugate (glucuronide) using highly sensitive and robust multiple reaction monitoring (MRM) quantitative analytical methods on UPLC–MS/MS machine.

In the modern era, more people are aspiring to become fit and healthy. Therefore, in addition to the aforementioned effects, intake of emodin health foods and slimming products [15] has increased due to its weight loss property through its traditionally known stimulant laxative activity. As described previously, the metabolism of emodin *in vitro* was gender different in rats and in four other animal species [7]. However, no study has been done to show the gender differences in oral bioavailability of emodin in rodents and humans. Hence, the main aim of our investigation is to study gender-dependent difference in PK profiles of emodin.

The purposes of this study are: (1) to develop a sensitive and reliable UPLC–MS/MS method to determine emodin concentration using in vivo single dose (8 mg/kg) oral and intravenous (4 mg/kg) PK model; (2) to study pharmacokinetic behavior of emodin and its metabolite in rats; and (3) to compare the gender difference in oral bioavailability of emodin in SD rats.

## 2. Experimental

### 2.1. Chemicals and reagents

Emodin (≥98%, HPLC grade) was purchased from Chengdu Mansite Pharmaceutical Company (China). Daidzein (≥98%, HPLC grade; internal standard, IS) was purchased from Sigma (St. Louis, MO, USA). PEG300 and Oral Suspensions Vehicle were purchased from PCCA laboratories (Houston, TX, USA). The experimental extraction procedures for emodin 3-*O*-β-D-glucuronide were essentially the same as previously published [7]. The 3D structure of emodin 3-*O*-β-D-glucuronide was confirmed by mass spectrometry and proton nuclear magnetic resonance spectroscopy ($^1$H NMR) [7]. All other materials (typically analytical grade or better) were used as received.

### 2.2. Instruments and conditions

An API 3200 Qtrap triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) was used to determine the plasma concentrations of emodin and emodin glucuronide. The quantitative analysis of emodin in aqueous and plasma medium was conducted using the MRM method in negative ion mode. The main working parameters for mass spectrometry were set as follows: ionspray voltage, 4.5 kV; ion source temperature, 350 °C; gas 1, 40 psi; gas 2, 20 psi; curtain gas, 10 psi. Daidzein was used as an internal standard in the quantification of emodin and emodin glucuronide in plasma. Emodin and emodin 3-*O*-β-D-glucuronide were determined via the MRM method in negative mode. Quantification transitions were used: emodin at $m/z$ 269 → $m/z$ 224.9 with collision energy set to 40 V, emodin 3-*O*-β-D-glucuronide at $m/z$ 445 → $m/z$ 268.6 with collision energy set to 30 V, and daidzein (IS) at $m/z$ 253 → $m/z$ 132 with collision energy set to 52 V. Dwell time was all set to 150 ms.

UPLC conditions for analyzing emodin in plasma were as follows: system, Waters Acquity™ with diode array detector (DAD); column, Acquity UPLC BEH C18 column (50 mm × 2.1 mm i.d., 1.7 mm; Waters, Milford, MA, USA); mobile phase B, 100% acetonitrile; mobile phase A, 100% aqueous buffer (2.5 mM $NH_4Ac$, pH 7.4); flow rate, 0.45 ml/min; wavelength, 254 nm for emodin and its glucuronide, and daidzein as an internal standard because the chemical is stable under UPLC conditions, with a consistent peak shape; injection volume, 10 μl; and column temperature, 45 °C. The UPLC conditions (mobile phase gradients) are shown in Table 1, while chromatograms and mass spectra in Fig. 2.

### 2.3. Pharmacokinetic study in vivo

#### 2.3.1. Animals

Male Sprague–Dawley (SD) rats (250–300 g, 8–10 weeks old) and female SD rats (200–230 g, 8–10 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN) and kept in an environmentally controlled room (temperature: 25 ± 2 °C, humidity: 50% ± 5%, 12 h dark–light cycle) for at least 1 week before the experiments. Treatment of the animals was permitted by the Ethics Committee of Southern Medical University.

#### 2.3.2. Pharmacokinetic experiments

Male and female SD rats were assigned into two groups. The animals were fasted overnight before drug administration. Emodin was freshly prepared just before i.v. administration by dissolving in 80% $NaHCO_3$ solution (pH 9.0) and 20% PEG300 at a dose of 4 mg/kg. After the resulting solution was filtered through 0.22 μm membrane, it was injected into the rat tail vein. For oral administration, emodin was dispersed in oral suspending vehicle and was given to rats at a dose of 8 mg/kg. Blood samples were withdrawn from tail vein at 5, 10, 15, 30, 60, 120, 240, 480, 720, and 1440 min after injection administration and at 5, 15, 30, 60, 120, 240, 480, 720, and 1440 min after oral administration. Each blood sample was cen-

**Table 1**
The mobile phase condition of UPLC for emodin and its glucuronide.

| | Mobile phase B | Mobile phase A |
|---|---|---|
| 0.1 min | 15% | 85% |
| 1.8 min | 40% | 60% |
| 2.2 min | 60% | 40% |
| 2.8 min | 15% | 85% |
| 3.2 min | 15% | 85% |

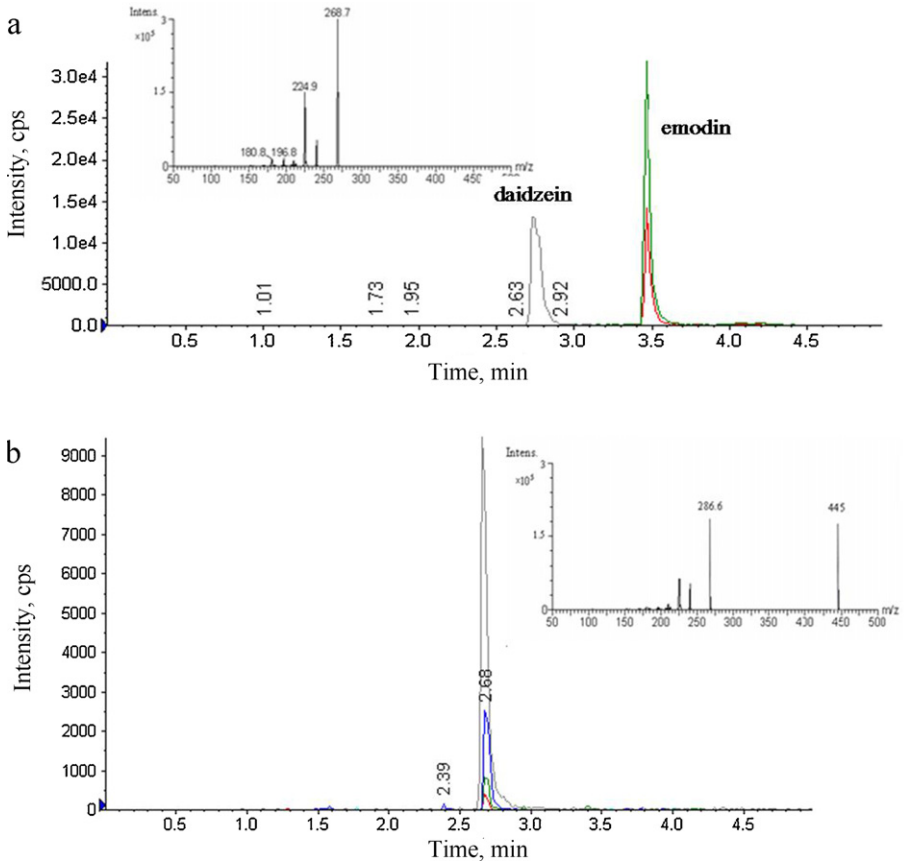


**Fig. 2.** Sample chromatograms of different analytes in current UPLC system with MS detection. A blank plasma sample spiked with (a) emodin (10 000 ng/ml) and daidzein (5000 ng/ml); (b) emodin 3-*O*-β-D-glucuronide (10 μM).

trifuged at 5000 rpm for 5 min. Afterwards, 100 μl of plasma was mixed with 320 μl of methanol containing 50 μM daidzein, and the mixture was centrifuged at 13 000 rpm for 30 min. The supernatant was then removed and dried under nitrogen gas. The residue was reconstituted in 100 μl methanol, vortexed, and then analyzed using UPLC–MS/MS.

*2.3.3. Preparation of standard and quality control samples*

Emodin stock solution was prepared in methanol at 2 mg/ml. The daidzein stock solution was prepared in methanol at 100 μg/ml. Calibration standard samples for emodin were prepared in blank rat plasma at 39, 78, 156, 312.5, 625, 1250, 2500, 5000, and 10 000 ng/ml. Three quality control (QC) samples were prepared as low (39 ng/ml), medium (625 ng/ml), and high (10 000 ng/ml) concentrations in the same way as the plasma sample preparation for calibration. The QC samples were stored at −20 °C until analysis. Calibration standard samples for emodin glucuronide were prepared in blank rat plasma at 78, 156, 312.5, 625, 1250, 2500, 5000, and 10 000 ng/ml. Three QC samples for emodin glucuronide were prepared as low (78 ng/ml), medium (625 ng/ml), and high (10 000 ng/ml) concentrations, in the same way as the plasma sample preparation for calibration. The QC samples were stored at −20 °C until analysis.

*2.3.4. Method validation*

*2.3.4.1. Calibration curve and LLOQ.* Calibration curves were prepared according to Section 2.3.3. The linearity of each calibration curve was confirmed by plotting the peak area ratio of emodin to IS in rat plasma. The $1/x$ least-squares linear regression method was used to determine the slope, intercept, and correlation coefficient of linear regression equation. LLOQ (the lower limit of quantification) was determined based on signal-to-noise ratio of 10:1.

*2.3.4.2. Precision and accuracy.* The "intraday" and "interday" precisions and accuracies of emodin were determined with three different concentrations of six QC samples in the same day or on three different days.

*2.3.4.3. Extraction recovery.* The extraction recovery of emodin was determined by comparing (a) the peak areas obtained from blank plasma spiked with analytes before the extraction with (b) those from samples to which analytes were added after the extraction.

*2.3.4.4. Stability.* Short-term (25 °C for 24 h), long-term (−20 °C for 90 days), and three freeze–thaw cycle stabilities of emodin were determined.

*2.3.5. Pharmacokinetic data analysis*

In the oral and intravenous PK studies, emodin was rapidly metabolized into emodin-3-*O*-β-D-glucuronide, the only metabolite of emodin observed via UPLC–MS/MS. The PK parameters for 8 mg/kg oral and intravenous emodin PK studies in male and female rats were calculated using WinNonlin 3.3 software. Total emodin plasma concentration values were calculated by adding the plasma concentrations of emodin and emodin glucuronide for each rat. The area under the concentration–time curve (AUC) was calculated using the trapezoidal rule with extrapolation to time infinity for total emodin, emodin aglycone, and emodin glucuronide. The absolute oral bioavailability was calculated as $F = [(AUC_{0-t,p.o.})/(AUC_{0-t,i.v.})] \times (Dose_{i.v.}/Dose_{p.o.}) \times 100\%$, where $AUC_{0-t,p.o.}$ and $AUC_{0-t,i.v.}$ correspond to the area under the

**Table 2**
Intra-day and inter-day precision and accuracy for emodin and emodin 3-*O*-β-D-glucuronide in MRM mode of UPLC–MS/MS analysis.

| Analyte | Concentration (ng/ml) | Intra-day ($n=6$) | | Inter-day ($n=6$) | |
|---|---|---|---|---|---|
| | | Accuracy (Bias, %) | Precision (CV, %) | Accuracy (Bias, %) | Precision (CV, %) |
| E | 39 | 90.46 | 6.42 | 89.23 | 7.93 |
| | 625 | 98.34 | 4.98 | 96.32 | 5.44 |
| | 10 000 | 104.75 | 2.83 | 102.09 | 4.72 |
| E-3-G | 78 | 92.45 | 3.89 | 95.98 | 7.52 |
| | 625 | 101.13 | 4.62 | 99.68 | 9.13 |
| | 10 000 | 105.85 | 4.83 | 102.43 | 6.74 |

E: emodin; E-3-G: emodin 3-*O*-β-D-glucuronide.

curve from zero to infinity after oral and i.v. administration, respectively.

### 2.4. Statistical analysis

The data in this paper are presented as mean ± SD, unless specified otherwise. Significance differences were assessed using unpaired Student's *t*-test with *P* values <0.05 considered statistically significant.

## 3. Results and discussion

### 3.1. Chromatography and mass spectrometry

A reliable and sensitive method to determine emodin concentration was established. The LLOQ of emodin was 20 ng/ml using UPLC–MS/MS method, which was more than 100 times lower than the published HPLC method by Shia et al. [14] to quantify emodin. In our study, methanol was used to precipitate rat plasma protein, which improved recovery. This newly established method enabled us to determine the concentration of emodin in vivo after administration of a low dose of emodin. Typical chromatograms of spiked emodin and emodin glucuronide in rat plasma sample are shown in Fig. 2a and b, respectively. MS2 spectra of these two chemicals were inserted in each chromatogram.

### 3.2. Linearity, precision and accuracy, recovery, matrix effect, and stability

The standard curve for emodin in plasma was linear in the concentration range of 39–10 000 ng/ml, with correlation coefficient values >0.998 (the weight is $1/x$). The LLOQ was 20 ng/ml, defined as a signal-to-noise ratio of ≥10. The standard curve for emodin glucuronide in plasma was linear in the concentration range of 78–10 000 ng/ml, with correlation coefficient values > 0.999 (the weight is $1/x$). The LLOQ was 40 ng/ml. Intra-day and inter-day precision and accuracy were determined by measuring six replicates of QC samples (emodin and its glucuronide) at three concentration levels in rat plasma. The precision and accuracy are shown in Table 2. The results showed that the precision and accuracy values were well within the 10% acceptance range.

The mean extraction recoveries determined using three replicates of QC samples of emodin at three concentration levels in rat plasma were 88.6% ± 4.85%, 92.3% ± 5.77%, and 97.9% ± 8.45% for 39, 625, and 10 000 ng/ml, respectively. The mean extraction recoveries determined using three replicates of QC samples of emodin glucuronide at three concentrations were 92.2% ± 6.21%, 96.3% ± 5.75%, and 97.9% ± 8.01% for 78, 625, and 10 000 ng/ml, respectively.

**Table 3**
Short-term stability, long-term stability and freeze–thaw cycles of emodin in rat plasma ($n=3$).

| Concentration (ng/ml) | Recovery% (mean ± SD) | | |
|---|---|---|---|
| | Short-term stability | Long-term stability | Freeze–thaw cycle |
| 39 | 102.74 ± 3.70 | 100.32 ± 9.15 | 91.34 ± 13.29 |
| 625 | 104.64 ± 6.93 | 92.45 ± 8.33 | 102.43 ± 10.93 |
| 10 000 | 99.83 ± 4.78 | 94.65 ± 6.89 | 102.53 ± 9.63 |

The stability of emodin in plasma was evaluated by analyzing three replicates of quality control samples containing 39, 625, and 10 000 ng/ml emodin after short-term storage (25 °C, 4 h), long-term cold storage (−20 °C, 90 days), and within three freeze–thaw cycles. All the samples displayed 95–105% recoveries after various stability tests, as shown in Table 3.

In earlier studies, Shia et al. [14] developed a HPLC method to analyze emodin and emodin glucuronide in plasma samples. The method was not sensitive enough to detect the plasma concentrations of emodin and emodin glucuronide up to 24 h. Therefore, a new highly sensitive UPLC–MS/MS method was developed and validated. The method showed significantly lower limit of quantification of 20 ng/ml, with signal to noise ratio of 10:1. The inter- and intra-day precisions for emodin and emodin glucuronide analysis were always within the acceptable range of 10%. The validated method was successfully used to analyze rat plasma samples after 8 mg/kg single dose oral and i.v. administration of emodin in male and female rats.

### 3.3. Pharmacokinetic studies

Plasma male rats showed 4-fold higher plasma concentrations of total emodin than female rats after 8 mg/kg oral single dose of emodin administration, suggesting a significant gender-dependent difference (Fig. 3 and Table 4). The observed gender difference in total emodin oral PK is due to higher AUC values of emodin glucuronide (more than 5-fold), not emodin aglycone, in male rats (Fig. 4 and Table 5). Similar to AUC, the $C_{max}$ values for emodin and emodin glucuronide were also higher in male (7-fold and 3.5-fold, respectively) than female rats.

The plasma concentration profile of emodin and emodin glucuronide after 4 mg/kg emodin intravenous administration in male

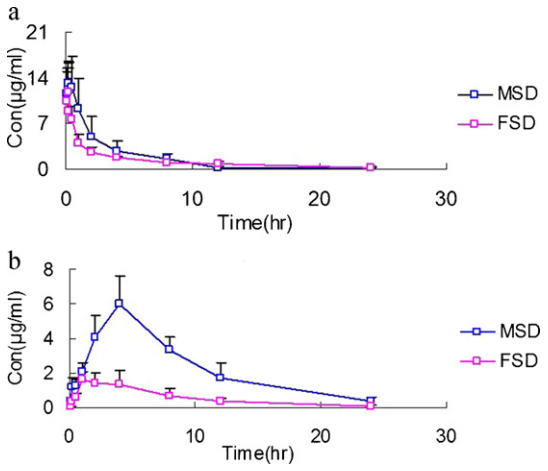


**Fig. 3.** Plasma concentration–time curves. (a) Total emodin (emodin + emodin glucuronide) after i.v. administration of emodin in SD male and female rats (4 mg/kg); (b) total emodin (emodin + emodin glucuronide) after oral dose of 8 mg/kg emodin in SD male and female rats (8 mg/kg). Each point represents an average of six determinations and the error bars are standard deviations of the mean.

**Table 4**
Pharmacokinetic parameters of total emodin (emodin + glucuronide) after oral and i.v. administration ($n = 6$).

| Parameters | i.v. | | Oral | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| $T_{max}$ (min) | 15.12 ± 4.97 | 15.04 ± 5.78 | 226.56 ± 51.82 | 62.08 ± 20.07 |
| $C_{max}$ (μg/ml) | 13.10 ± 3.45 | 11.87 ± 3.58 | 5.99 ± 1.61 | 1.59 ± 0.59 |
| $AUC_{0-t}$ (min μg/ml) | 2133.47 ± 734.49 | 1839.08 ± 446.09 | 3034.59 ± 968.99 | 762.07 ± 321.89 |
| $t_{1/2}$ (min) | 311.10 ± 47.65 | 533.08 ± 210.57 | 304.29 ± 94.63 | 346.52 ± 114.53. |
| $MRT_{inf}$ (min) | 190.48 ± 67.62 | 311.25 ± 106.54 | 392.82 ± 132.87 | 341.18 ± 145.26 |

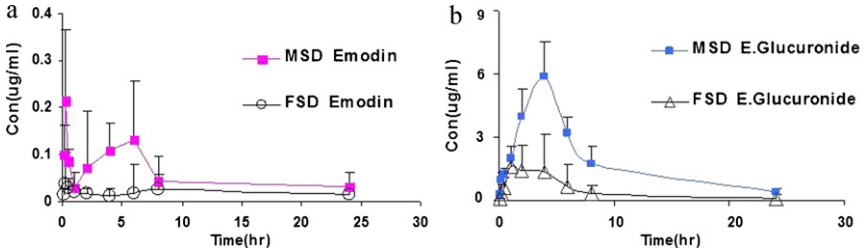


**Fig. 4.** Plasma concentration–time curves. (a) Emodin after oral administration in SD male and female rats (8 mg/kg); (b) emodin glucuronide after oral administration of emodin in SD male and female rats (8 mg/kg). Each point represents an average of six determinations and the error bars are standard deviations of the mean.

**Table 5**
Pharmacokinetic parameters of emodin and emodin 3-*O*-β-D-glucuronide after oral administration ($n = 6$).

| Parameters | E | | E-3-G | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| $T_{max}$ (min) | 18.00 ± 6.71 | 18.75 ± 7.51 | 240 ± 0.00 | 60 ± 0.00 |
| $C_{max}$ (μg/ml) | 0.21 ± 0.094 | 0.039 ± 0.011 | 5.89 ± 1.64 | 1.81 ± 2.58 |
| $AUC_{0-t}$ (min μg/ml) | 79.93 ± 31.52 | 33.82 ± 4.09 | 2661.89 ± 635.87 | 458.50 ± 373.29 |
| $t_{1/2}$ (min) | 385.02 ± 103.25 | 1593.23 ± 954.22 | 330 ± 154.62 | 319.35 ± 148.29 |
| $MRT_{inf}$ (min) | 519.09 ± 153.93 | 623.03 ± 172.31 | 476.55 ± 124.84 | 327.96 ± 241.24 |

E: emodin; E-3-G: emodin 3-*O*-β-D-glucuronide.

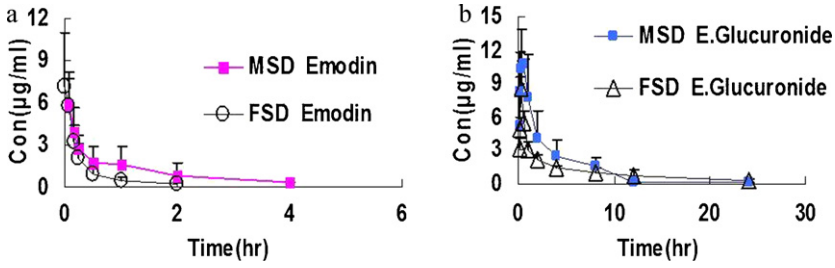


**Fig. 5.** Plasma concentration–time curves. (a) Emodin after i.v. administration in SD male and female rats (4 mg/kg); (b) emodin glucuronide after i.v. administration of emodin in SD male and female rats (4 mg/kg). Each point represents an average of six determinations and the error bars are standard deviations of the mean.

and female SD rats showed no gender-dependent difference (Fig. 5). Emodin aglycone plasma concentrations declined biexponentially and were only detectable within 4 h in both the male and the female rats. Furthermore, emodin glucuronide was detected in plasma only 5 min after the i.v. administration of emodin in both male and female rats. The PK parameters of emodin and emodin glucuronide after 4 mg/kg intravenous administration of emodin in male and female rats are listed in Table 6. A gender-dependent significantly higher oral systemic bioavailability of total emodin in male than in female SD rats has been shown for the first time.

Significantly higher oral systemic bioavailablity of total emodin in male than female rats (Figs. 3 and 4) may be explained by higher intestinal emodin absorption and lower intestinal excretion of emodin glucuronide in male than female SD rats [7]. Furthermore,

**Table 6**
Pharmacokinetic parameters of emodin and its metabolite (emodin 3-*O*-β-D-glucuronide) after intravenous injection ($n = 6$).

| Parameters | E | | E-3-G | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| $T_{max}$ (min) | 5.0 ± 2.62 | 10 ± 5.51 | 30 ± 10.64 | 15 ± 6.04 |
| $C_{max}$ (μg/ml) | 5.83 ± 2.34 | 7.19 ± 3.23 | 10.74 ± 4.62 | 8.61 ± 2.41 |
| $AUC_{0-t}$ (min μg/ml) | 430.82 ± 110.33 | 258.64 ± 98.73 | 1813.71 ± 463.76 | 1410.42 ± 312.07 |
| $t_{1/2}$ (min) | 82.5 ± 35.66 | 63.00 ± 12.47 | 306.64 ± 137.83 | 560.27 ± 109.65 |
| $MRT_{inf}$ (min) | 65.37 ± 23.12 | 50.58 ± 22.45 | 198.74 ± 22.14 | 361.03 ± 101.13 |

E: emodin; E-3-G: emodin 3-*O*-β-D-glucuronide.

the male rat intestinal and liver microsome studies previously published by our lab has also shown that the rate of glucuronidation of emodin was significantly higher in male than female S–D rats at lower, as well as higher, concentrations in the liver and intestine [7]. This higher rate of metabolism may also cause apparent increase in the intestinal absorption in male rats if excreted faster, thereby causing increased oral bioavailability of total emodin (especially emodin glucuronide) in male than female rats.

In another study, i.v. administration of emodin did not shows any gender-dependent difference in total systemic bioavailability of emodin, as well as individual systemic bioavailability of emodin and emodin glucuronide in male and female rats. This suggests that the observed gender difference in absolute oral bioavailability of total emodin might not be due to the difference in the elimination pathway of emodin and emodin glucuronide in male and female rats.

## 4. Conclusion

A rapid, sensitive, and specific UPLC–MS/MS method was developed, validated, and successfully applied for quantifying emodin in rat plasma samples. A 4-fold gender-dependent difference was observed in the absolute oral bioavailability of total emodin in male (1.6%) and female (0.4%) SD rats.

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