

Research Article

Development and Validation of an UHPLC-MS/MS Method for Quantification of DMAG in Rat Plasma and Its Application in a Preliminary Pharmacokinetic Study in Thrombocytopenia Rats

Yan Li ^{1,2}, Yuqing Wang,² Zhiqiang Zhao,^{2,3} Yunxia Li ¹, Cheng Peng,¹
and Jianming Wu ²

¹State Key Laboratory of Southwestern Chinese Medicine Resources, Key Laboratory of Standardization for Chinese Herbal Medicine, Ministry of Education; School of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, China

²Institute of Cardiovascular Research, The Key Laboratory of Medical Electrophysiology, Ministry of Education, Medical Key Laboratory for Drug Discovery and Druggability Evaluation of Sichuan Province, Luzhou Key Laboratory of Activity Screening and Druggability Evaluation for Chinese Materia Medica, School of Pharmacy, Southwest Medical University, Luzhou 646000, China

³School of Pharmacy, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

Correspondence should be addressed to Yan Li; 993603736@qq.com and Jianming Wu; jianmingwu@swmu.edu.cn

Received 28 February 2023; Revised 7 August 2023; Accepted 19 August 2023; Published 2 September 2023

Academic Editor: Damião Pergentino de Sousa

Copyright © 2023 Yan Li et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

A preliminary study has shown that 3, 8-Di-O-methyllellagic acid 2-O-glucoside (DMAG), an ellagic tannin from *Sanguisorba officinalis* L., has the potential in relieving thrombocytopenia. However, there is a lack of information on the pharmacokinetics of DMAG in thrombocytopenia rats. Therefore, we aimed to establish a simple, rapid, and sensitive UHPLC-MS/MS method for quantifying DMAG and study its pharmacokinetic behavior in this study. DMAG and hispidulin (internal standard, IS) were separated on an Acquity Shim-pack GIST column using 0.1% formic acid in water and acetonitrile as the mobile phase with a total run time of 5 min and gradient elution at a flow rate of 0.3 mL/min. The recovery and matrix effects of DMAG were within 94.85%–100.40% and 94.55%–102.46%, respectively. The intraday RSD and interday RSD were between 3.31% and 13.19%, accuracy RE was $\leq 6.69\%$, and stability RSD changes were 3.38%–8.78%. As for intragastric administration, with shortened T_{max} (3.00 vs. 2.16 h), C_{max} (25.67 vs. 35.38 ng/mL) was added for a 2 mg/kg dose after the establishment of the thrombocytopenia rat model. Relative to normal rats treated with 4 mg/kg, in thrombocytopenia rats treated with the same dose, C_{max} (49.13 vs. 67.78 ng/mL), $AUC_{(0-t)}$ (234.60 vs. 318.17 ng·h/mL), and $AUC_{(0-\infty)}$ (322.74 vs. 498.57 ng·h/mL) increased, MRT (18.15 vs. 26.32 h) prolonged, T_{max} (3.00 vs. 2.33 h) shortened, and CL/F (12746.50 vs. 8093.50 mL/h/kg) reduced. As for intravenous administration, C_{max} (1679.54 ng/mL), $AUC_{(0-t)}$ (589.02 g·h/mL), and $AUC_{(0-\infty)}$ (605.58 g·h/mL) were significantly increased in thrombocytopenia rats than that in normal rats (743.76 ng/mL for C_{max} , 242.46 g·h/mL for $AUC_{(0-t)}$, and 245.19 g·h/mL for $AUC_{(0-\infty)}$). V_z/F and CL/F were remarkably decreased from 343196.86 to 194659.43 mL/kg for V_z/F and 8236.18 to 3326.01 mL/h/kg for CL/F with the model establishment, respectively. Overall, we successfully developed a reliable UHPLC-MS/MS method for determining DMAG levels in rat plasma. The pharmacokinetic difference could be attributed to the pathological state of the thrombocytopenia rats, which may affect the absorption, distribution, metabolism, and excretion of DMAG. These findings lay the foundation for further evaluating the clinical efficiency and safety of DMAG in the treatment of thrombocytopenia.

1. Introduction

Sanguisorba officinalis L. is an herbal medicine from the Rosaceae family that can be found in both north and south

China. It has been reported to have various beneficial effects, such as hemostatic, antitumor, antioxidative, antibacterial, and anti-inflammatory [1]. One of the most common clinical applications of *Sanguisorba officinalis* L. is as a hemostatic

agent. It has been used to treat various conditions, such as upper gastrointestinal hemorrhages, ulcer hemorrhages, hematochezia, metrorrhagia, and metrostaxis. Moreover, it has been studied for its potential in the treatment of leukemia, hepatic and gastric carcinoma, and scalds [2]. Our research group has primarily focused on studying the mechanism of hematopoiesis induced by *Sanguisorba officinalis* L. for a considerable period of time. Previous studies conducted by our group have demonstrated that the extract of *Sanguisorba officinalis* L. can stimulate bone marrow hematopoiesis by promoting the proliferation and differentiation of hematopoietic stem and progenitor cells, ultimately leading to an increase in the number of leukocytes, erythrocytes, and platelets [3, 4].

Tannins, flavonoids, and saponins are primary chemical components in *Sanguisorba officinalis* L. [5, 6]. Tannins, one of the primary components, can be divided into three categories: hydrolyzable, condensed, and complex tannins formed by glucoside bond formation between condensed and hydrolyzable tannins. Hydrolyzable tannins can be further categorized into gallic acid tannins and ellagitannins based on the type of esterified polyols and phenolic acids produced during the hydrolysis process. DMAG, a typical ellagic tannin, exhibits a wide range of effects, including antitumor, antioxidation, antiseptic, and immune regulation [7, 8]. Our previous research has demonstrated that it can enhance megakaryocyte differentiation, platelet production, aggregation, and adhesion, resulting in a reduction in tail bleeding time. The underlying mechanism of these effects may involve the upregulation of p-Akt, VWF, PLEK, ITGB3, and ITGA2B expression [9]. Therefore, DMAG is a potential candidate for the treatment of thrombocytopenia. However, the pharmacokinetic characteristics of DMAG in the plasma of normal and thrombocytopenic rats have not been reported at present.

Currently, various analytical methods have been employed to determine the active components in *Sanguisorba officinalis* L. For instance, Xu et al. identified 3, 3'-di-O-methyl ellagic acid and 3, 3', 4'-trimethyl ellagic acid in *Polygonum runcinatum* using HPLC [10]. Xiong and Yang employed HPLC to investigate the changes in pharmacokinetic characteristics of catechin, epicatechin, and ziyuglycoside I in rats after compatibility with different tannins and saponins. When the compatibility proportion reached 8:1, the three components exhibited favorable pharmacokinetic behavior in rats [11]. Sun et al. employed HPLC-ELSD to detect triterpenoids and phenolic acids in *Sanguisorba officinalis* L. [12]. Tang et al. used HPLC-MS/MS to simultaneously determine the pharmacokinetic characteristics of ziyuglycoside I and II in rats [13]. Fan et al. conducted a study using HPLC-MS/MS to detect and analyze thirteen phenolic acids and six triterpenoids in the *n*-butanol extract of *Sanguisorba officinalis* L. [14]. They also compared the pharmacokinetic differences of these components between normal rats and rats with leukopenia. In a separate study, Wu et al. utilized UHPLC-MS/MS to analyze six triterpenoids [15]. Wei et al. used UHPLC-MS/MS to measure the pharmacokinetic parameters of three compounds in rats

after they were orally administered *Sanguisorba officinalis* L. extract [16]. Zhu et al. employed UHPLC-MS/MS to detect ziyuglycoside I and compared its pharmacokinetic behavior in normal and leukopenia rats [17]. However, to the best of our knowledge, there is currently no reported analytical method specifically designed to detect DMAG in thrombocytopenia rats.

This present study aims to establish an UHPLC-MS/MS method that is simple, rapid, and sensitive for determining DMAG. The pharmacokinetic characteristics of DMAG will be studied, and the difference in pharmacokinetics between normal and thrombocytopenic rats will be compared. The findings of this study will provide reference information for the development of innovative drugs for treating thrombocytopenia.

2. Materials and Methods

2.1. Chemicals and Reagents. DMAG (98% purity), IS (98% purity), and cyclophosphamide (98% purity) were purchased from BioBioPha Co., Ltd. (Yunnan, China), Chengdu Mansite Pharmaceutical Co., Ltd. (Chengdu, China), and Meryer Chemical Technology Co., Ltd. (Shanghai, China), respectively. The chemical structures of DMAG and IS are shown in Figure 1. Quick Start Bradford 1x Dye Kit and TIANamp Genomic DNA Kit were purchased from Bio-Rad Laboratories (California, USA) and Tiangen Biotech Co., Ltd. (Beijing, China), respectively. Formic acid and acetonitrile (LC/MS grade) were acquired from Thermo Fisher Scientific Co., Ltd. (USA). Distilled deionized water was prepared using a Plus Milli-Q water purification system (Bedford, MA, USA). Ethyl acetate was of analytical grade supplied by Aladdin Reagents Co., Ltd. (Shanghai, China).

2.2. Animals and Model Preparation. Female and male specific pathogen-free Sprague Dawley rats, weighing 180–220 g (Certificate no. SCXK 2020-030), were provided by Da-suo Biotechnology Co., Ltd. (Chengdu, China). These rats were housed for one week in a standard breeding room (temperature 22–25°C, humidity 55 ± 10%, and 12 h light/12 h dark cycle) and allowed free access to food and water. All animal experiments were conducted following US guidelines [18] and approved by the Ethical Committee on Use and Care of Animals of Chengdu University of Traditional Chinese Medicine (No. 2015-0727-03). Before the experiment, the rats were fasted for 12 h with free access to water.

The rats were then randomly divided into normal and model groups. A rat model for thrombocytopenia induced by cyclophosphamide was established in our previous study [19]. The blood of six normal rats and six model rats was collected for platelet count. Bone marrow cells were extracted from rat femur to calculate the bone marrow cell count with an automatic blood cell analyzer and protein content and DNA content according to kit instructions. In the meantime, the heart, liver, spleen, lung, kidney, and thymus indices of the normal and model groups were collected and determined.

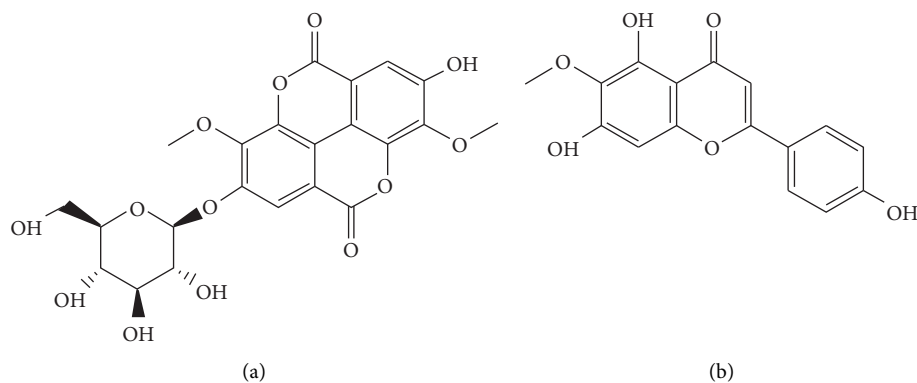


FIGURE 1: The chemical structures of DMAG (a) and IS (b).

2.3. Chromatographic Condition. A UHPLC-MS/MS system (Shimadzu Corp., Tokyo, Japan) equipped with an LC-MS triple quadrupole mass spectrometer was used to quantify DMAG and IS. Chromatographic separation was achieved on an Acquity Shim-pack GIST column (2.0 × 75 mm, 2.0 μm; Shimadzu Corp., Japan) at 40°C. The mobile phase was composed of 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.3 mL/min: 0–2.00 min, 40% to 60% B; 2.01–3.00 min, 60% to 40% B; 3.01–5.00 min, and the ratio of B maintained at 40%.

The mass spectrometer with an ESI ion source was operated in the negative ion mode. The MRM fragmentation transitions were m/z 491.35 → 313.10 for DMAG and m/z 299.05 → 284.10 for IS (Figure 2). The parameters of mass spectrometry were optimized: flow rates of atomizing gas, 2.0 L/min; heating gas, 10.0 L/min; drying gas, 10.0 L/min; interface voltage, 4 kV; interface temperature, 300°C; desolvation temperature, 250°C; heating block temperature, 400°C; and detector voltage, 2.24 kV. The collision energies of DMAG and IS were 35 eV and 19 eV, respectively.

2.4. Preparation of Standard Solutions, Calibration, and Quality Control (QC) Samples. Stock solutions of DMAG and IS were prepared with acetonitrile at 0.20 and 25 mg/mL, respectively. The calibration curve working solution of DMAG was prepared by diluting the stock solution with 0.1% formic acid aqueous: acetonitrile = 60:40 (mobile phase) to achieve a series of concentrations: 0.5, 4, 20, 50, 100, 200, 500, and 1000 ng/mL. IS stock solutions were diluted into 400 ng/mL with the mobile phase. The calibration standard was prepared by spiking 90 μL blank rat plasma with 10 μL working standard solutions. QC samples were prepared at 0.5, 20, 100, and 500 ng/mL with the same operation.

2.5. Plasma Sample Preparation. A 10 μL aliquot of IS was added to 100 μL rat plasma. After mixing for 30 s, 3.5 mL of ethyl acetate was added for drug extraction. Then, the mixture was vortexed for 5 min and centrifuged at 8000 g for 10 min at 4°C. The supernatant was collected, dried under nitrogen flow at 37°C, and reconstituted with 100 μL mobile phase. A 10 μL aliquot was injected immediately into the UHPLC-MS/MS spectrometer for analysis.

2.6. Method Validation. According to the FDA guidelines [20–22], the bioanalytical method by UHPLC-MS/MS technique was developed, and specificity, recovery, matrix effect, accuracy, precision, linearity, and stability of the method should be validated.

2.6.1. Specificity. The specificity of the method was assessed by analyzing the chromatograms of six different batches of blank rat plasma, plasma samples with DMAG and IS, and plasma samples after DMAG administration.

2.6.2. Linearity. For the calibration curve, a series of concentrations (0.05, 0.4, 2, 5, 10, 20, 50, and 100 ng/mL) were prepared for DMAG in rat plasma. Also, the concentration of IS was 40 ng/mL in rat plasma. To evaluate the linearity, the calibration curve of DMAG was constructed by plotting the peak area ratios (y) of DMAG to IS against the theoretical concentrations (x) and fitted to linear regression analysis. The lower limit of quantification (LLOQ) was sufficient for the pharmacokinetic study of DMAG in rat plasma with a signal-to-noise ratio (SNR) ≥ 10. In the meantime, the accuracy (relative error, RE) and precision (relative standard deviation, RSD) were permitted to be ≤ 20%.

2.6.3. Matrix Effect and Recovery. The matrix effect and recovery of rat plasma samples were determined at quality control (QC) concentrations (2, 10, and 50 ng/mL, $n = 6$) by comparing the following samples: (A) DMAG and IS were added into plasma before extraction, (B) both DMAG and IS were added into the residue after extracting blank plasma, and (C) DMAG and IS were directly added into acetonitrile. Matrix effects were evaluated by calculating the peak area ratio of DMAG and IS with or without plasma after extraction at three QC concentrations (group B/group C). The recovery was assessed by comparing the peak area ratio of DMAG and IS before and after treatment with ethyl acetate (group A/group B).

2.6.4. Accuracy and Precision. Accuracy and precision were evaluated using QC samples of LLOQ, low, medium, and high concentration on one day and three consecutive days.

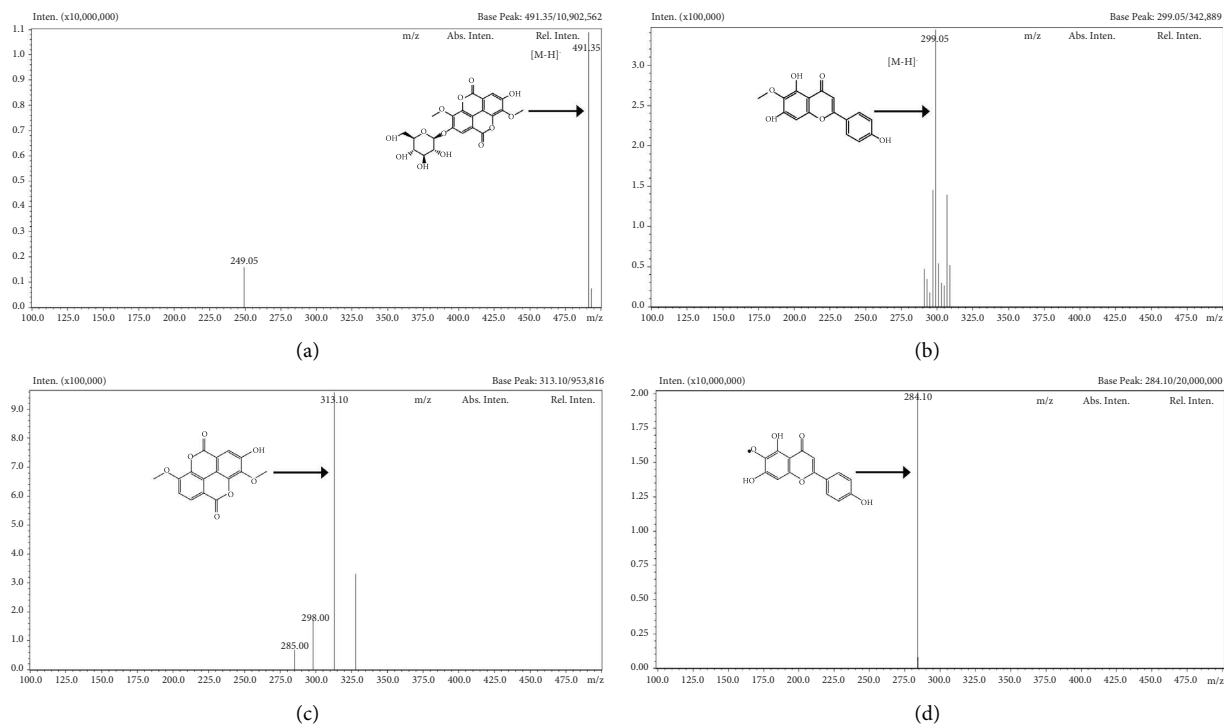


FIGURE 2: The MS/MS spectrums of DMAG (a) and IS (b), and main corresponding product ion of DMAG (c) and IS (d).

Accuracy was presented as the relative error (RE, %) between measured concentrations and actual concentrations. And precision was exhibited as relative standard deviation (RSD, %). Accuracy and precision for low, medium, and high concentrations should be less than 15%, and LLOQ not exceed 20%.

2.6.5. Stability. The stability of the analyte under various storage and handling conditions was calculated using three concentrations of QC samples, which included the short-term (12 h, 25°C), long-term (30 d, -80°C), postpreparative (24 h, 4°C), and three freeze-thaw cycles (-80°C) stabilities. Then, the stability of the analyte in rat plasma was assessed by analyzing six replicates.

2.7. Pharmacokinetics. Twelve normal rats and twelve model rats were randomly divided into low- and high-dose groups (three female and three male rats in each group). The low- and high-dose groups were orally administered with 2 and 4 mg/kg DMAG, respectively. In addition, six normal rats and six model rats (three female and three male rats in each group) were intravenously administered one dose of 2 mg/kg DMAG. A series of blood samples were acquired before and after oral administration at time points of 0.08, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, and 48 h. Also, blood samples were centrifuged at 1500 g for 10 min to obtain plasma samples and then kept at -80°C.

2.8. Data Analysis. Pharmacokinetic parameters, including AUC_{0-t} , $AUC_{0-\infty}$, MRT, T_{max} , V_z/F , CL/F , and C_{max} , were calculated with DAS 3.0 software. Simultaneously,

experimental data were analyzed using GraphPad Prism 8.0 or SPSS 22.0. When the two groups of samples followed the normal distribution and homogeneity of variance, a *t*-test was used; otherwise, nonparametric tests were used. $P < 0.05$ or $P < 0.01$ was considered statistically significant.

3. Results and Discussion

3.1. Thrombocytopenia Rat Model Validation. Cyclophosphamide is a potent alkylating agent that can form covalent bonds with DNA molecules, leading to the inhibition of DNA synthesis in marrow hematopoietic cells. This disruption of DNA synthesis affects the balance between cell proliferation and maturation of megakaryocytes, and platelet senescence. As a result, cyclophosphamide can cause a decrease in platelet levels in the peripheral blood, a condition known as thrombocytopenia [23]. Consequently, cyclophosphamide is commonly used to establish rat models of thrombocytopenia. Compared with normal rats, DNA content, protein content, platelet count, bone marrow cell count, spleen index, and thymus index in model rats were all reduced (Supplementary Figure 1S).

3.2. Method Development and Validation. Pharmacokinetics is the scientific study of how drugs are absorbed, distributed, metabolized, and eliminated by the body. It focuses on understanding the movement of drugs within the body over time, including factors such as the drug's absorption into the bloodstream, its distribution to various tissues and organs, its metabolism by enzymes, and its elimination from the body through urine or feces [24]. By studying pharmacokinetics, researchers can gain insights into how drugs are

processed by the body, which can help optimize drug dosing regimens and improve drug safety and efficacy. To judge the application prospects of a drug, especially its marketability, it not only has to have strong curative effects, minimal toxicity, and side effects but also excellent pharmacokinetic properties. The most typical examples are peptide drugs. Many bioactive peptides in the body, such as endorphins, which are high efficiency and low toxicity, are unstable in the body with poor absorption [25]. Pharmacokinetic studies in the early stages of drug design and development will help obtain safe and effective therapeutic drugs and reduce the elimination rate of candidate drugs. Nevertheless, no pharmacokinetic study of DMAG in rats has been reported till date. In the present study, a novel, accurate, sensitive, and efficient UHPLC-MS/MS method was established to determine the pharmacokinetics of DMAG in rats *in vivo*. To improve the ionization efficiency and peak shape of DMAG, an aqueous solution containing 0.1% formic acid and acetonitrile was selected as the mobile phase in gradient elution for analysis. Liquid-liquid extraction with ethyl acetate was adopted because of its excellent recovery efficiency for DMAG.

Specificity was assessed by comparing the MRM chromatograms of blank plasma samples, blank plasma samples with DMAG and IS, and plasma samples after the administration of DMAG (Figure 3). As shown in the figure, the retention times of DMAG and IS were 1.068 and 2.000 min, respectively. No endogenous substances interfered with DMAG and IS detection, which proved its high specificity. As shown in Table 1, recovery and matrix effects of DMAG were within the ranges of 94.85%–100.40% and 94.55%–102.46%, respectively. The intra- and interday precision and accuracy of DMAG at QC concentrations are shown in Table 2. The intraday RSD and interday RSD were between 3.31% and 13.19%, and the accuracy RE was $\leq 6.69\%$, which proved that the analytical method was accurate and reproducible. A calibration curve was established by plotting the peak area ratio of DMAG and IS to the concentration. Linearity was good in the concentration range of 0.05–100 ng/mL. The regression equation for DMAG was $y = 0.00600871x + 0.00011248$ ($r^2 = 0.9994$). The lower limit of quantitation (LLOQ) for DMAG was 0.05 ng/mL. The stability of DMAG in rat plasma under different storage conditions is shown in Table 3, with stability RSD changing in the range of 3.38%–8.78%. These results indicated that DMAG was sufficiently stable under the above analytical conditions.

3.3. Pharmacokinetic Study. The mean plasma concentration-time profiles of DMAG in rat plasma after intragastric administration are shown in Figure 4. Pharmacokinetic parameters of DMAG in rats after intragastric administration in the four groups are presented in Table 4. For normal and model rats, C_{\max} , $AUC_{(0-t)}$, and $AUC_{(0-\infty)}$ increased between the two dose groups. After establishing the thrombocytopenia rat model, T_{\max} was reduced (3.00 vs. 2.16 h), and C_{\max} was increased (25.67 vs. 35.38 ng/mL) at a dose of 2 mg/kg. Relative to normal rats, in thrombocytopenia rats treated with 4 mg/kg, C_{\max} (49.13 vs. 67.78 ng/mL), $AUC_{(0-t)}$ (234.60 vs. 318.17 ng h/mL), and $AUC_{(0-\infty)}$

(322.74 vs. 498.57 ng h/mL) were increased, MRT (18.15 vs. 26.32 h) was prolonged, T_{\max} (3.00 vs. 2.33 h) was clearly shortened, and CL/F (12746.50 vs. 8093.50 mL/h/kg) was reduced. Furthermore, mean plasma concentration-time profiles of normal and thrombocytopenia rats after intravenous administration are exhibited in Figure 5. Pharmacokinetic parameters of DMAG in rats after intravenous administration are manifested in Table 5. Although no difference can be observed for MRT and $t_{1/2}$ between two groups, C_{\max} (1679.54 ng/mL), $AUC_{(0-t)}$ (589.02 g-h/mL), and $AUC_{(0-\infty)}$ (605.58 g-h/mL) were significantly increased in thrombocytopenia rats than that in normal rats (743.76 ng/mL for C_{\max} , 242.46 g-h/mL for $AUC_{(0-t)}$, and 245.19 g-h/mL for $AUC_{(0-\infty)}$). V_z/F and CL/F were remarkably decreased from 343196.86 to 194659.43 mL/kg for V_z/F and 8236.18 to 3326.01 mL/h/kg for CL/F with the model establishment, respectively. The results showed that the absorption of DMAG was increased, T_{\max} advanced, and elimination slowed in thrombocytopenia rats, signifying that the pharmacokinetic behavior of DMAG may be affected by the pathological state. Also, it is consistent with the relevant literature report. For example, the AUC, C_{\max} , and $t_{1/2}$ of protosappanin B and Brazilian were increased in diabetic rats when compared to normal rats after oral administration of the extract from *Sappan Lignum* [26]. Compared to the normal group, the exposure to paeoniflorin, albiflorin, and plumbagin in liver injury rats was enhanced, while their elimination was slowed down [27]. With decreased CL, AUC, C_{\max} , and $t_{1/2}$ of aloe-emodin, rhein, emodin, and chrysophanol almost doubled after administration of the extract of *Rheum palmatum* L. in thrombotic focal cerebral ischemia rats compared to normal rats [28].

The pharmacokinetic alteration of DMAG may be also influenced by the presence of intestinal flora. These bacteria, specifically *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, and *Clostridium hathewayi*, are capable of producing trimethylamine, which can induce the concentration of endogenous trimethylamine oxide (TMAO) [29]. When platelets are directly exposed to TMAO, it can increase the release of stored calcium ions, leading to the activation of platelets in response to stimuli [30]. In addition, intestinal flora can promote platelet adhesion, aggregation, and activation through stimulation of Toll-like receptor 2, regulation of von Willebrand factor. Intestinal flora can also facilitate the production of intestinal endotoxins, which stimulate the secretion of endothelial cytokine VIII and are associated with the occurrence and development of thrombocytopenia [31, 32]. Furthermore, studies have shown that ellagic tannins can increase the diversity of intestinal flora in normal rats and alter the composition of the flora by increasing the proportion of *Bacteroidales* S24-7, *Lachnospiraceae* NK4A136, and *Bacteroides* [33]. Gallic acid tannins, when administered tannins from *Sanguisorba officinalis* L., can be hydrolyzed by intestinal flora to produce gallic acid, which can further be metabolized to pyrogallol. However, due to their large molecular weight and high polarity, ellagic tannins have poor absorption and low bioavailability in the gastrointestinal tract of mammals, making it difficult for them to reach bioactive concentrations

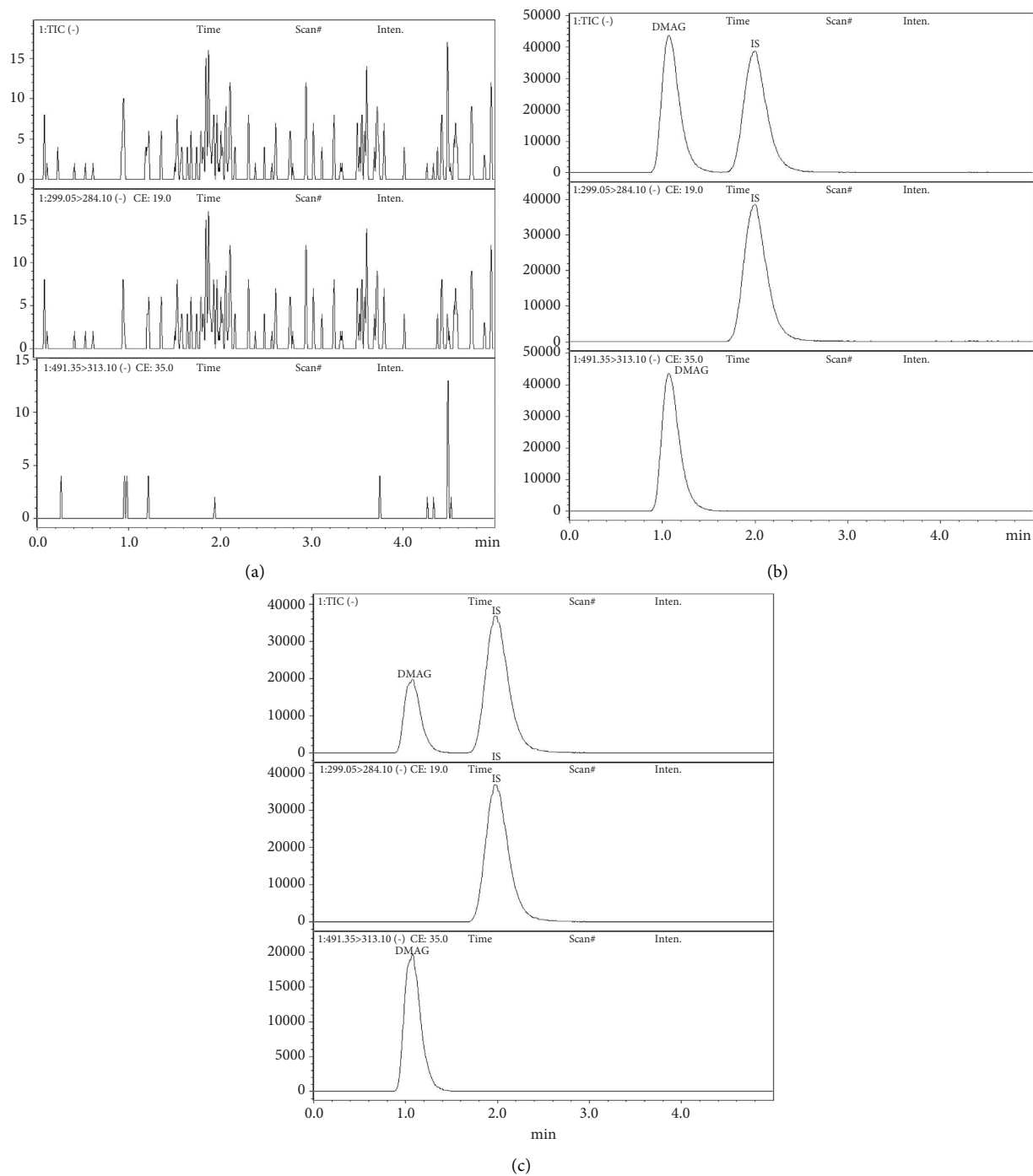


FIGURE 3: The MRM chromatograms of blank plasma (a), blank plasma with 10 ng/mL DMAG and 40 ng/mL IS (b), and (c) plasma collected at 0.5 h after treated with 2 mg/kg DMAG in thrombocytopenia rat.

TABLE 1: Recovery and matrix effect of DMAG (% , $n=6$).

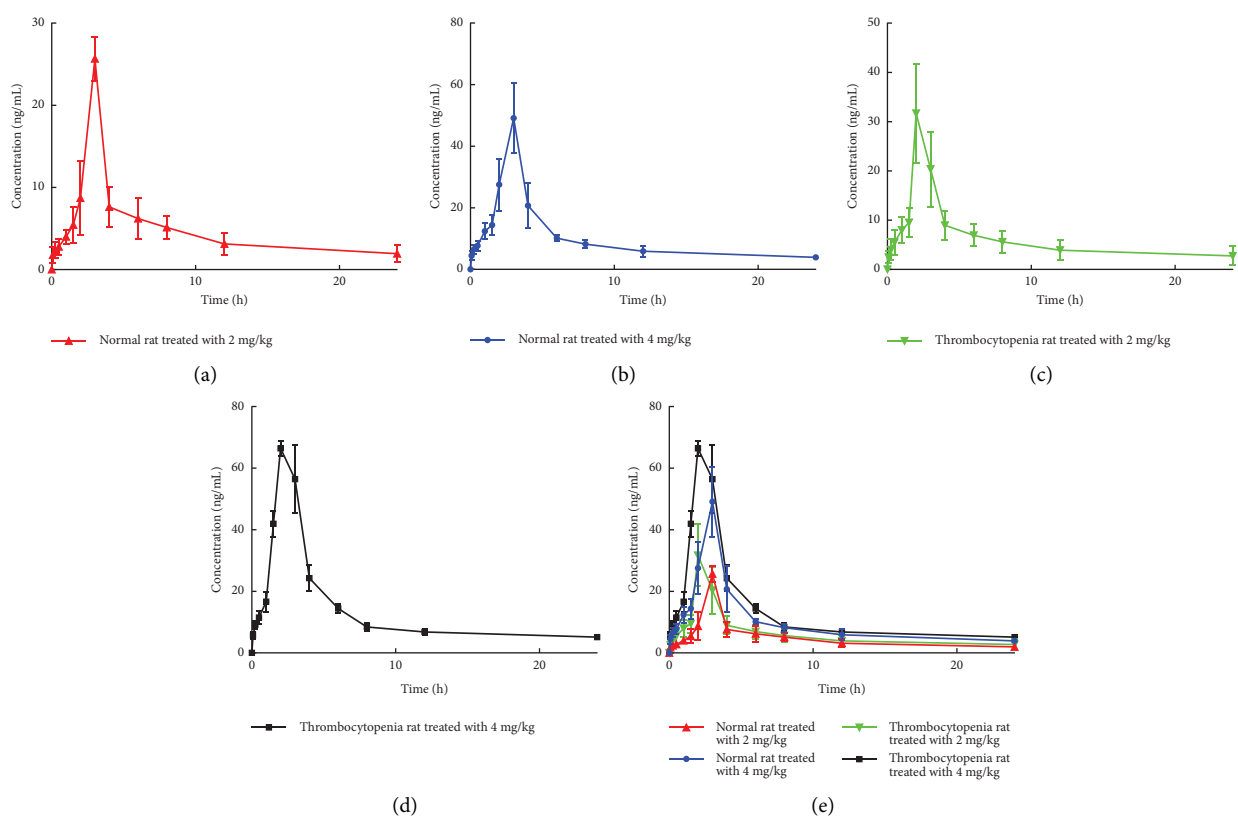
Concentration (ng/mL)	Recovery	Matrix effect
2.00	94.85 ± 9.17	102.46 ± 5.72
10.00	100.40 ± 5.16	94.55 ± 8.86
50.00	99.41 ± 3.57	95.88 ± 4.94

TABLE 2: Precision and accuracy for intraday and interday of DMAG ($n = 6$).

Concentration (ng/mL)	Intraday			Interday		
	Mean \pm SD	Accuracy (RE %)	Precision (RSD %)	Mean \pm SD	Accuracy (RE %)	Precision (RSD %)
0.05	0.04 \pm 0.01	-0.17	11.80	0.04 \pm 0.01	-0.42	13.19
2.00	1.88 \pm 0.16	-5.79	8.55	1.92 \pm 0.08	-3.59	4.64
10.00	10.67 \pm 0.35	6.69	3.31	10.47 \pm 0.86	4.76	8.19
50.00	47.70 \pm 4.33	-4.59	9.08	49.56 \pm 2.35	-0.86	4.75

TABLE 3: Stability of DMAG under different storage conditions (RSD %, $n = 6$).

Concentration (ng/mL)	Short-term (12 h, 25°C)	Plasma sample (24 h, 4°C)	Long-term (30 d, -80°C)	Three freeze-thaw cycles (-80°C)
2.00	5.13	3.69	8.01	6.17
10.00	4.95	8.78	3.56	5.72
50.00	4.71	3.38	6.43	7.09

FIGURE 4: Mean plasma concentration-time profiles of normal rats after intragastric administration of 2 (a) and 4 mg/kg (b) DMAG, thrombocytopenia rats after intragastric administration of 2 (c) and 4 mg/kg (d) DMAG, and the integrated plasma concentration-time profiles of each group (e) ($n = 6$).

in tissues and plasma. Some of the unabsorbed ellagellin is hydrolyzed to ellagic tannins, which then lose a lactone ring under the action of intestinal flora. These tannins are gradually dehydroxylated or methylated into more lip-soluble and absorbable urolithins. Urolithins have high biological activity and may be the active components *in vivo* [34, 35]. In summary, there is an interaction between ellagic tannins and intestinal flora, and the presence of intestinal

flora may cause pharmacokinetic changes in DMAG. However, further research is needed to confirm whether DMAG can be absorbed by intestinal flora and converted to urolithin during intestinal metabolism, as well as how it regulates the intestinal flora in thrombocytopenia rats.

In addition, while establishing the pathological model of thrombocytopenia, we also considered the drug-drug interactions between cyclophosphamide and DMAG. This

TABLE 4: Pharmacokinetic parameter of DMAG in rat after intragastric administration of normal and thrombocytopenia rats ($n=6$).

Parameters	Normal rat		Thrombocytopenia rat	
	2 mg/kg	4 mg/kg	2 mg/kg	4 mg/kg
AUC_{0-t} (ng·h/mL)	114.58 ± 20.97	234.60 ± 31.26**	148.05 ± 43.72	318.17 ± 24.49 ^{▲▲##}
$AUC_{0-\infty}$ (ng·h/mL)	146.58 ± 54.06	322.74 ± 56.46**	237.45 ± 138.32	498.57 ± 52.66 ^{▲▲##}
MRT (h)	14.95 ± 9.25	18.15 ± 4.64	22.92 ± 13.94	26.32 ± 6.76 [▲]
$t_{1/2}$ (h)	10.95 ± 7.35	15.34 ± 4.02	18.58 ± 11.53	24.84 ± 6.23
T_{max} (h)	3.00 ± 0.00	3.00 ± 0.00	2.16 ± 0.40**	2.33 ± 0.51 [▲]
V_z/F (mL/kg)	210756.33 ± 91244.18	274150.16 ± 50417.19	228507.00 ± 88363.40	286188.33 ± 50910.10
CL/F (mL/h/kg)	14799.66 ± 3889.18	12746.50 ± 2436.63	10303.66 ± 4027.34	8093.50 ± 806.26 ^{▲▲}
C_{max} (ng/mL)	25.67 ± 2.68	49.13 ± 11.40**	35.38 ± 2.99**	67.78 ± 2.46 ^{▲▲##}

AUC_{0-t} , area under the concentration-time curve from 0 to 24 h; $AUC_{0-\infty}$, area under the concentration-time curve from 0 to time infinite; MRT, mean residence time; $t_{1/2}$, half-life of elimination; T_{max} , time to achieve maximum concentration; V_z/F , volume of distribution; CL/F, clearance; C_{max} , maximum value of concentration; ** $P < 0.01$ significant compared to normal rat treated with 2 mg/kg, [▲] $P < 0.05$, ^{▲▲} $P < 0.01$ compared to normal rat treated with 4 mg/kg, ^{##} $P < 0.01$ compared to thrombocytopenia rat treated with 2 mg/kg.

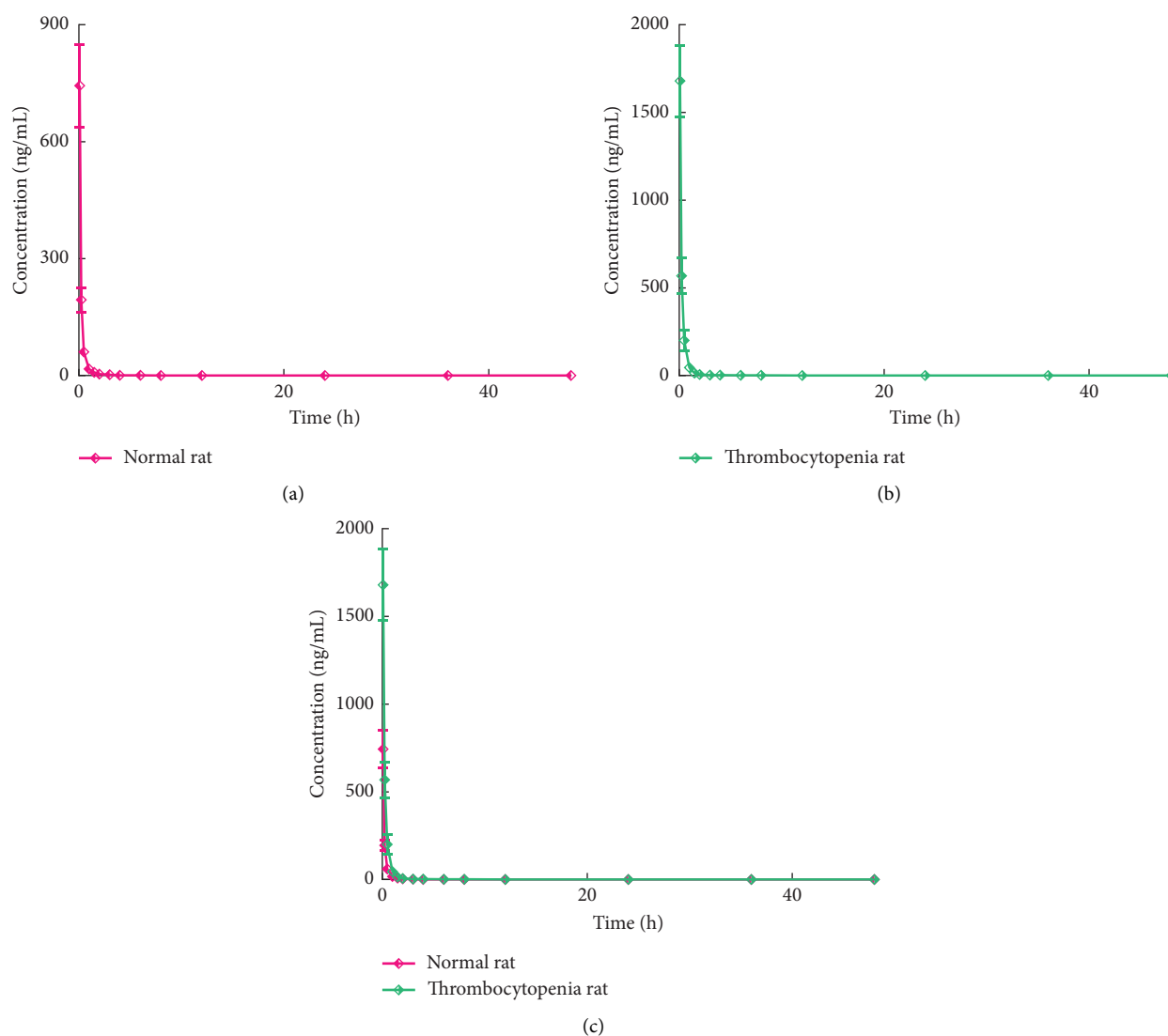


FIGURE 5: Mean plasma concentration-time profiles of normal rats (a) and thrombocytopenia rats (b) after intravenous administration of 2 mg/kg DMAG, and the integrated plasma concentration-time profiles of each group (c) ($n=6$).

TABLE 5: Pharmacokinetic parameter of DMAG in rat after intravenous administration of normal and thrombocytopenia rats with 2 mg/kg ($n = 6$).

Parameter	Normal rat	Thrombocytopenia rat
AUC _{0-t} (ng·h/mL)	242.46 ± 27.01	589.02 ± 63.39**
AUC _{0-∞} (ng·h/mL)	245.19 ± 27.48	605.58 ± 56.15**
MRT (h)	1.79 ± 0.39	4.19 ± 2.30
$t_{1/2}$ (h)	29.10 ± 8.36	39.84 ± 10.40
V_z/F (mL/kg)	343196.86 ± 97272.04	194659.43 ± 66689.18*
CL/F (mL/h/kg)	8236.18 ± 854.77	3326.01 ± 303.26**
C_{max} (ng/mL)	743.76 ± 106.11	1679.54 ± 204.07**

AUC_{0-t}, area under the concentration-time curve from 0 to 48 h; AUC_{0-∞}, area under the concentration-time curve from 0 to time infinite; MRT, mean residence time; $t_{1/2}$, half-life of elimination; V_z/F , volume of distribution; CL/F, clearance; C_{max} , maximum value of concentration; * $P < 0.05$ or ** $P < 0.01$ significant compared to normal rat.

interaction could be related to the changes in the pharmacokinetics of DMAG. Therefore, it is important to evaluate the potential drug-drug interaction between cyclophosphamide and DMAG. Previous research has shown that both cyclophosphamide and tannin are substrates for CYP3A4 [36–38], which could explain the decreased clearance and extended mean residence time of DMAG. Other studies have also found that the levels of *Lactobacillus* and *Bifidobacterium* were reduced, while *Enterococcus* and *Escherichia coli* were increased in the intestinal tract of mice treated with cyclophosphamide. This could be one of the reasons for the variations in the absorption of DMAG [39, 40].

Tumors are neoplasms formed by the excessive proliferation of local tissue cells in the body under the effect of various tumorigenic factors. Tumor cells can adapt to change in the metabolic environment by transforming between oxidative phosphorylation (OXPHOS) and glycolysis, making them extremely difficult to eliminate. Traditional therapies for tumors include surgery, radiation, and chemotherapy. However, the current most commonly used means of tumor treatment, chemoradiotherapy, not only kills cancer cells but also normal cells. This can result in bone marrow suppression and severe side effects such as leukopenia, erythrocytopenia, and thrombocytopenia, thus limiting its scope and efficacy [41]. Among these side effects, thrombocytopenia is a common cause of clinical bleeding with spontaneous and intracranial hemorrhage, which is life-threatening. The pathogenesis is complex, has not been fully elucidated, and is a major problem that has puzzled clinicians for a long time. Therefore, in the process of chemoradiotherapy, it is of paramount importance to protect the hematopoietic function of the bone marrow and improve the platelet count in the peripheral blood [42]. A relevant study has shown that tannins from *Sanguisorba officinalis* can inhibit proliferation and promote apoptosis of SMMC-7721 and HepG2 cells [43, 44]. Qin et al. found that the survival rate of human cancer cell lines such as A549, SGC-7901, and BEL7402 gradually decreased with increasing concentration of total saponins from *Sanguisorba officinalis* L. [45]. Bastow et al. reported that Sanguin H-6 had a strong inhibitory effect on DNA

topoisomerase, with IC₅₀ of 1 $\mu\text{mol/L}$ and 0.01 $\mu\text{mol/L}$ for topoisomerase I and II *in vitro*, respectively. The IC₅₀ of Sanguin H-6 on HeLa25 cells was 12 $\mu\text{mol/L}$ and showed a dose-response relationship [46]. Consequently, *Sanguisorba officinalis* L. can be simultaneously used to treat tumors and thrombocytopenia. DMAG, a typical ellagic tannin from *Sanguisorba officinalis* L., is expected to be developed as an innovative candidate with this dual effect, which requires further experimental verification.

4. Conclusions

This study presents the first bioanalytical method for quantifying and studying the pharmacokinetics of DMAG in rat plasma using UHPLC-MS/MS. Additionally, significant differences in the pharmacokinetic characteristics of normal and thrombocytopenia rats were observed after oral and intravenous administration, potentially due to the pathological state. These findings provide valuable insights into the pharmacokinetic behavior of DMAG and contribute to our understanding of its effectiveness and toxicity.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Li and Wang contributed equally to this paper.

Acknowledgments

This study was supported by the National Major Science and Technology Project of the Ministry of Science and Technology of China (Grant no. 2018ZX09721004-006-004), National Natural Science Foundation of China (Grant nos. 81774013, 81804221, and 82074129), Science and Technology Planning Project of Sichuan Province, China (Grant no. 2022NSFSC1451), Science and Technology Program of

Luzhou, China (Grant nos. 2019LZXNYDJ11 and 2018-JYJ-34), Xinglin Scholar Research Promotion Project of Chengdu University of Traditional Chinese Medicine (Grant no. MPRC2022018), and Science and Technology Programs of Southwest Medical University (Grant no. 2019ZZD006).

Supplementary Materials

Supplementary Figure 1S: DNA content, protein content, platelet count, bone marrow cell count, spleen index, and thymus index of normal and model rats. (*Supplementary Materials*)

References

- [1] W. J. Zhang, C. Peng, X. Shen et al., "A bioactive compound from *Sanguisorba officinalis* L. inhibits cell proliferation and induces cell death in 5-fluorouracil-sensitive/resistant colorectal cancer cells," *Molecules*, vol. 26, p. 3843, 2021.
- [2] J. A. Shin, J. S. Kim, K. H. Kwon et al., "Apoptotic effect of hot water extract of *Sanguisorba officinalis* L. in human oral cancer cells," *Oncology Letters*, vol. 4, pp. 489–494, 2012.
- [3] J. H. Yang, M. Yang, J. Huang, and R. Y. Zhang, "Preparation of burnet tannins and preliminary study of its influence on leukopenia in mice induced by cyclophosphamide," *Chin Med Biotechnol.*, vol. 8, pp. 41–45, 2013.
- [4] X. Chen, B. G. Li, Y. Gao, J. X. Ji, Z. L. Wu, and S. Chen, "Saponins from *Sanguisorba officinalis* improve hematopoiesis by promoting survival through FAK and Erk1/2 activation and modulating cytokine production in bone marrow," *Frontiers in Pharmacology*, vol. 8, p. 130, 2017.
- [5] X. X. Meng, Y. F. Xian, L. Xiang et al., "Complete chloroplast genomes from *Sanguisorba*: identity and variation among four species," *Molecules*, vol. 23, p. 2137, 2018.
- [6] C. F. Bai, Y. S. Sun, X. C. Pan et al., "Antitumor effects of trimethyllellagic acid isolated from *Sanguisorba officinalis* L. on colorectal Cancer via angiogenesis inhibition and apoptosis induction," *Frontiers in Pharmacology*, vol. 10, p. 1646, 2020.
- [7] M. Oliviero, M. Stanzione, M. D. Auria, L. Sorrentino, S. Iannace, and L. Verdolotti, "Vegetable tannin as a sustainable UV stabilizer for polyurethane foams," *Polymers*, vol. 11, p. 480, 2019.
- [8] Y. Guo, K. Sakulnarmrat, and I. Konczak, "Anti-inflammatory potential of native Australian herbs polyphenols," *Toxicology Reports*, vol. 1, pp. 385–390, 2014.
- [9] J. Lin, J. Zeng, S. Liu et al., "DMAG, a novel countermeasure for the treatment of thrombocytopenia," *Molecular Medicine*, vol. 27, p. 149, 2021.
- [10] C. F. Xu, J. Y. Huang, Y. Hao, L. Li, Q. Liu, and M. Zhang, "Determination of 3, 3'-di-O-methyl ellagic acid and 3, 3', 4'-trimethyl ellagic acid in *Polygonum runcinatum* by HPLC," *West China Journal of Pharmaceutical Sciences*, vol. 30, pp. 230–231, 2015.
- [11] Y. A. Xiong and M. Yang, "Effects of *sanguisorba* tannins and saponins compatibility on pharmacokinetic parameters of catechin, epicatechin and ziyuglycoside I in rats," *China Journal of Chinese Materia Medica*, vol. 41, pp. 3661–3667, 2016.
- [12] J. H. Sun, C. L. Gan, J. Huang et al., "Determination of triterpenoids and phenolic acids from *Sanguisorba officinalis* L. by HPLC-ELSD and its application," *Molecules*, vol. 26, p. 4505, 2021.
- [13] J. Tang, J. Zhang, W. Sun et al., "Simultaneous determination of Ziyuglycoside I and Ziyuglycoside II in rat plasma by liquid chromatography–electrospray ionization mass spectrometry and its application in a pharmacokinetic study," *Chin J Bioprocess Eng.*, vol. 17, pp. 536–541, 2019.
- [14] L. Z. Fan, X. T. Wang, J. Huang et al., "Comparison of the pharmacokinetic profiles of 13 phenolic acids and 6 triterpenes in normal and leukopenia rats after oral administration of *Sanguisorba officinalis* L. extract by LC–MS/MS," *Journal of Separation Science*, vol. 43, pp. 4103–4122, 2020.
- [15] C. Wu, M. C. Yao, W. Li et al., "Simultaneous determination and pharmacokinetics study of six triterpenes in rat plasma by UHPLC–MS/MS after oral administration of *sanguisorba officinalis* L. extract," *Molecules*, vol. 23, p. 2980, 2018.
- [16] F. Wei, C. Yang, L. Wu, J. Sun, Z. Wang, and Z. Wang, "Simultaneous determination and pharmacokinetics study of three triterpenes from *Sanguisorba officinalis* L. In rats by UHPLC–MS/MS," *Molecules*, vol. 27, p. 5412, 2022.
- [17] L. J. Zhu, L. Chen, C. F. Bai et al., "A rapid and sensitive UHPLC–MS/MS method for the determination of ziyuglycoside I and its application in a preliminary pharmacokinetic study in healthy and leukopenic rats," *Biomedicine and Pharmacotherapy*, vol. 123, Article ID 109756, 2020.
- [18] National Academy Press, "Guide for the care and use of laboratory animals," *Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council*, National Academy Press, Washington, DC, USA, 8th edition, 2011.
- [19] Y. Q. Wang, J. M. Wu, Y. X. Li et al., "A Rapid and sensitive UHPLC–MS/MS method for determination of 2, 3, 8-trimethyllellagic, a potent active compound from *Sanguisorba officinalis* L. and its application in the pharmacokinetic study within thrombocytopenia rats," *Journal of Chemical Neuroanatomy*, vol. 2021, Article ID 3309434, 13 pages, 2021.
- [20] Center for Drug Evaluation and Research (CDER), *Guidance for Industry on Bioanalytical Method Validation*, Center for Drug Evaluation and Research (CDER), Rockville, MD, USA, 2018.
- [21] R. A. Xu, Q. Lin, X. Qiu et al., "UPLC-MS/MS method for the simultaneous determination of imatinib, voriconazole and their metabolites concentrations in rat plasma," *Journal of Pharmacy Biomedicine Analytical*, vol. 166, pp. 6–12, 2019.
- [22] C. Tang, X. Niu, L. Shi, H. Zhu, G. Lin, and R. A. Xu, "In vivo pharmacokinetic drug-drug interaction studies between fedratinib and antifungal agents based on a newly developed and validated UPLC/MS-MS method," *Frontiers in Pharmacology*, vol. 11, Article ID 626897, 2020.
- [23] L. X. Chen, Y. L. Qi, Z. Qi et al., "A comparative study on the effects of different parts of *Panax ginseng* on the immune activity of cyclophosphamide-induced immunosuppressed mice," *Molecules*, vol. 24, p. 1096, 2019.
- [24] Y. Y. Qi, Q. C. Zhang, and H. X. Zhu, "Huang–Lian Jie–Du decoction: a review on phytochemical, pharmacological and pharmacokinetic investigations," *Chinese Medicine*, vol. 14, p. 57, 2019.
- [25] S. W. Bruun, K. Josefsen, J. T. Tanassi et al., "Large gliadin peptides detected in the pancreas of NOD and healthy mice following oral administration," *Journal of Diabetes Research*, vol. 2016, Article ID 2424306, 11 pages, 2016.
- [26] X. Z. Tong, H. Zhu, Y. Shi, H. T. Xu, B. Wang, and J. H. Zhao, "An LC/MS/MS method for simultaneous quantitation of two homoisoflavones: protosappanin B and brazilin with hypoglycemic activity in rat plasma and its application to a comparative pharmacokinetic study in normal and

- streptozotocin-treated rats," *Journal of Ethnopharmacology*, vol. 148, pp. 682–690, 2013.
- [27] F. J. Jiang, Y. L. Zhao, J. B. Wang et al., "Comparative pharmacokinetic study of paeoniflorin and albiflorin after oral administration of Radix Paeoniae Rubra in normal rats and the acute cholestasis hepatitis rats," *Fitoterapia*, vol. 83, pp. 415–421, 2012.
- [28] S. X. Feng, J. S. Li, L. B. Qu, Y. M. Shi, and D. Zhao, "Comparative pharmacokinetics of five Rhubarb anthraquinones in normal and thrombotic focal cerebral ischemia-induced rats," *Phytotherapy Research*, vol. 27, pp. 1489–1494, 2013.
- [29] T. V. Kirichenko, Y. V. Markina, V. N. Sukhorukov, V. A. Khotina, W. K. Wu, and A. N. Orekhov, "A novel insight at atherogenesis: the role of microbiome," *Frontiers in Cell and Developmental Biology*, vol. 8, Article ID 586189, 2020.
- [30] J. L. Ma and H. K. Li, "The role of gut microbiota in atherosclerosis and hypertension," *Frontiers in Pharmacology*, vol. 9, p. 1082, 2018.
- [31] S. Jackel, K. Kiouptsi, M. Lillich, T. Hendriks, N. Hormann, and E. Wilms, "Gut microbiota regulate hepatic von Willebrand factor synthesis and arterial thrombus formation via Toll-like receptor-2," *Blood*, vol. 130, pp. 542–553, 2017.
- [32] I. Arbesu, M. Bucsaiova, M. B. Fischer, and C. Mannhalter, "Platelet-borne complement proteins and their role in platelet-bacteria interactions," *Journal of Thrombosis and Haemostasis*, vol. 14, pp. 2241–2252, 2016.
- [33] Q. B. Shen, S. J. Li, T. F. Liu et al., "Effects of ellagic acid on gut microbiota in SD rats evaluated by high-throughput sequencing," *J Anhui Agric Sci.*, vol. 47, pp. 171–173, 2019.
- [34] I. E. Sallam, A. Abdelwareth, A. Heba et al., "Effect of gut microbiota biotransformation on dietary tannins and human health implications," *Microorganisms*, vol. 9, p. 965, 2021.
- [35] C. Y. He, J. Fu, J. Y. Ma et al., "Biotransformation and *in vitro* metabolic profile of bioactive extracts from a traditional miao-nationality herbal medicine," *Polygonum capitatum. Molecules*, vol. 19, pp. 10291–10308, 2014.
- [36] B. H. Zhou, P. Chen, X. X. Guo, H. P. Zhang, Y. Wu, and G. Liu, "Effect of tannins from Pericarpium Granati on hepatic microsomal enzyme in rats," *China Pharmacy*, vol. 20, pp. 1517–1520, 2017.
- [37] J. Zhang, C. Wang, Q. Liu et al., "Pharmacokinetic interaction between JBP485 and cephalexin in rats," *Drug Metabolism and Disposition*, vol. 38, pp. 930–938, 2010.
- [38] Z. Huang, P. Roy, and D. J. Waxman, "Role of human liver microsomal CYP3A4 and CYP2B6 in catalyzing N-dechloroethylation of cyclophosphamide and ifosfamide," *Biochemical Pharmacology*, vol. 59, pp. 961–972, 2000.
- [39] R. Ecknauer and U. Löhns, "The effect of a single dose of cyclophosphamide on the jejunum of specified pathogenfree and germfree rats," *Digestion*, vol. 14, pp. 269–280, 1976.
- [40] T. Zuo, L. Cao, X. H. Sun et al., "Dietary squid ink polysaccharide could enhance SIgA secretion in chemotherapeutic mice," *Food and Function*, vol. 5, pp. 3189–3196, 2014.
- [41] P. P. Wang, F. M. An, X. J. Zhuang et al., "Chronopharmacology and mechanism of antitumor effect of erlotinib in lewis tumor-bearing mice," *PLoS One*, vol. 9, Article ID e101720, 2014.
- [42] H. Zhou, L. Liu, X. H. Shu, X. R. Wang, and Y. P. Song, "Clinical efficacy and safety of high-dose dexamethasone plus low-dose rituximab as first-line therapy in newly diagnosed primary immune thrombocytopenia," *Indian Journal of the Hematol Blood Transfus*, vol. 35, pp. 507–512, 2019.
- [43] Y. Hu, T. Xia, and J. B. Zhao, "Antitumor-effect of sanguisorba officinalis tannins on liver cancer cell smmc-7721 by mtt and fcm," *Journal of the Fourth Military Medical University*, vol. 19, pp. 550–552, 1998.
- [44] C. L. Wan, J. H. Chai, X. W. Sun, J. W. Hao, and X. F. Sun, "The effects and mechanisms of *n*-butanol extract from Sanguisorba Officinalis L. on the proliferation and apoptosis of human liver cancer HepG2 cells," *Anti-tumor Pharm.*, vol. 4, pp. 112–116, 2014.
- [45] S. H. Qin, K. Li, L. Zhou, Z. L. Hu, H. X. Zhao, and R. Zheng, "Experimental study on the antitumor effect of Sanguisorba saponins," *Shandong Medical Journal*, vol. 50, pp. 24–26, 2010.
- [46] K. F. Bastow, I. D. Bori, Y. Fukushima et al., "Inhibition of DNA topoisomerases by sanguin H-6, a cytotoxic dimeric ellagitannin from Sanguisorba officinalis," *Planta Medica*, vol. 59, pp. 240–245, 1993.