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Comparative Plasma Pharmacokinetics of Ziyuglycoside I from *Sanguisorba Officinalis L.* between Leukopenia and Normal Rats via UHPLC-MS/MS

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Summary

Objectives: Ziyuglycoside I (ZgI), one of the main active ingredients of Diyushengbai tablet made from Sanguisorba officinalis L., has been proved to relieve leukopenia. In our study, we compared the difference of pharmacokinetics of ZgI between normal and leukopenia rats.

Materials and methods: 24 rats were divided into four groups, low and high dose (orally taken ZgI 5 or 20 mg/kg, respectively) control or leukopenia groups, induced by intraperitoneal injection of 70 mg/kg Cyclophosphamide (CY) twice. plasma samples were collected from orbital venous plexus at 0, 5, 10, 20, 40, 60 min, 1.5, 2, 4, 8, 12, 24, 48 h after oral administrated of ZgI, and concentrations of ZgI were analyzed by Ultra-High Performance Liquid Chromatography-tandem Mass-spectrometry (UHPLC-MS/MS).

Results: Compared with20 mg/kg normal group, peak time (T_{max}) was significantly shortened (0.93 h to 0.33 h) and maximum Concentration (C_{max}) was remarkably decreased (7.96 ng/L to 3.40 ng/L) in 20 mg/kg leukopenia group, on the contrary, elimination half-life ($T_{1/2\beta}$) in it was obviously prolonged (5.02 h to 18.51 h). However, there were no clearly differences in distributed half-life ($T_{1/2\alpha}$) and area under the plasma concentration vs. time curve from zero to last sampling time (AUC_{0-t}) between20 mg/kg leukopenia and control group ($p>0.05$). All above changes were similar between 5 mg/kg model and control group, except C_{max} were nearly equal between them. Interestedly, there was also no evidently difference between the two leukopenia groups.

Conclusion: The pharmacokinetic process especially absorption and metabolism of ZgI were evidently influenced by leukopenia. Our study may provide guidance for clinical use of Diyushengbai tablet and development of ZgI as an agent for the treatment of leukopenia.

Keywords: Leukopenia; Pharmacokinetics; Sanguisorba officinalis L.; UHPLC-MS/MS; Ziyuglycoside I

Introduction

Diyushengbai Tablet (DST), a Chinese patent medicine made from raw material of *Sanguisorba officinalis L.*, has been used to cure leukopenia caused by various radiotherapy and chemotherapy in clinic for more than 20 years, which is the major side effect of stopping cancer treatment, and shown significant efficacy [1-3]. Ziyuglycoside I (ZgI), the main saponin from *Sanguisorba officinalis L.*, has been proved to be the primarily active ingredient of leukogenic action of DST [4]. Previous studies have shown that ZgI can remarkably increase the levels of White Blood Cells (WBCs) and platelets in leukopenia mice induced by Cyclophosphamide (CY) and can also relieve its bone marrow depression [5-7].

As we all known, pharmacokinetic study of a drug is an essential step for preclinical research and clinical trial and plays an important role in the process of innovative drug development and research [8]. However, there few literatures have been reported about the pharmacokinetic studies of ZgI during the past 20 years [9]. Recently years, a large number of studies have demonstrated that pharmacokinetics of drugs can be affected by many factors, including age, gender, altitude, drugs and variety of diseases, such as diabetes, Hypertension, estrogen level and so on [10-17]. Actually, drugs are used to treat diseases, and the patients are the ultimate consumers of drugs [18]. That is to say, study on drug pharmacokinetics in a disease state is more important than the normal condition and is more clinically relevant [19]. Therefore, we consider that the previous pharmacokinetic study of ZgI was not suitable for clinic as the animal model they used.

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In the present study, we established an UHPLC-MS/MS method to investigate the pharmacokinetics of ZgI in a rat model of leukopenia and further explored the difference to that of the normal for the first time. This study may provide a rationale for clinical use of Diyushengbai tablet and development of ZgI as an agent for the treatment of leukopenia.

Materials and Methods

Chemicals and reagents

ZgI (batch number: 111562-201312, purity > 98%) and Glycyrrhetinic Acid [GA, batch number: 110723-200612, purity > 98%, as Internal Standard (IS)] were supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and confirmed by a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). CY was purchased from Jiangsu Hengrui Pharmaceuticals Co. LTD (Lianyungang, Jiangsu, China). Ethyl acetate, ethanol, UHPLC-grade water, methanol, acetonitrile and ammonia were purchased from Tedia Company, Inc. (Shanghai, China). Distilled deionized water was freshly generated by a Plus Milli-Q water purification system (Bedford, MA, USA).

Animals

Male Sprague-Dawley (SD) rats (certificate number: SCXK2013-17, 8-10 weeks old and body weight 250-350 g) were provided by Southwest Medical University (Luzhou, Sichuan, China). The rats were housed in plastic cages (four rats per cage) in 12-h light/12-h dark cycle with free access to diet and water at a room temperature 25°C ± 1°C and humidity 60% ± 5%. The rats were fasted over night before experiment. All animal experiments were performed strictly in accordance with University guidelines and were approved (Permit No. 20160276) by the Committee on Use and Care of Animals of Southwest Medical University (Luzhou, Sichuan, China).

Establishment of leukopenia model in rats

The first step, rats were intraperitoneally (i.p.) injected with CY at 40, 70, 100 mg/kg to select the appropriate dose for establishing leukopenia model, while the control rats were i.p. injected with same volume of normal saline on the first and third days once a day for twice. Then, WBCs were counted from the blood of venous plexus of rats by an automated hematology analyzer XT-1800i (Sysmex Corporation, Bin County, Kobe city, Japan). Finally, choose the best dosage for induction of leukopenia in rats.

Instrumentation and analytical conditions

The UHPLC-MS/MS analysis was carried out on a Shimadzu Chromatographic System composed of LC-30AD × 2 infusion pumps, DGU-20A5 online degasser, SIL-30AC automatic sampling device, CTO-20A column oven, CBM-20A system controller, and a LCMS-8050 triple-quadrupole mass spectrometer. Lab Solutions LCMS Ver.5.85 software was used for data acquisition and analysis. UHPLC-separation was performed on a Shim-pack GIST column (2.1 mm I.D. × 50 mm l, 2.0 μm, P/N 227-30001-02), maintained at 40°C. The mobile phase consisted of H_2O with 0.05% ammonia (solvent A) and acetonitrile with 0.05% ammonia (solvent B). A binary gradient elution (flow rate at 0.45 mL/min) was performed for the separation, and the consecutive program was as follows: an isocratic elution of 10% solvent B for the initial 0.5 min, followed by a linear gradient elution of 10%-98% solvent B for the next 1.5 min, then holding the composition of 98% solvent B for the next 1 min, and finally column equilibration to the initial conditions over 2 min. The sample manager temperature was maintained at 10°C and the injection volume was 10 μL. Meanwhile, 20 μL of pure water was injected for the analysis of ZgI in order to improve the peak shape.

The mass spectrometer was handled in the negative pattern. Quantification was obtained using Multiple Reaction Monitoring (MRM) acquisition mode by monitoring the precursor ion to product ion transitions of m/z 765.45→m/z 603.50 for ZgI and m/z 469.15→355.10 for GA (IS). The ion spray voltage and source temperature were maintained at 3.5 kV and 400°C, respectively. DL temperature was 200°C. The collision energy for ZgI and IS were set at 51 eV and 52 eV, respectively. The atomizing gas, heating gas, and drying gas were 2.5 L/min, 8.0 L/min and 10.0 L/min, respectively.

Preparation of samples

Standard stock solutions of ZgI (1.07 mg/mL) and GA (1.05 mg/mL) were prepared by dissolving the accurate weight of reference substances in methanol. Intermediate stock solutions of ZgI (10.00 μg/mL) and GA (10.00 μg/mL) were prepared from the stand stock solutions. All of the solutions were stored at 4°C until detection. Sodium heparin was added into the plasma samples obtained from normal and leukopenia rats with or with treatments for anticoagulation and plasma samples without treatment was used as blank. The plasma samples were stored at -80°C until experiment.

Nine calibration samples and four Quality Control (QC) samples were prepared with appropriate amounts of the Intermediate Stock solutions of ZgI and GA (IS) in blank plasma. The concentrations of the calibration samples of ZgI in plasma were 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/mL. QC samples were prepared at four concentrations: 5 ng/mL (lower limit of quantification quality control, LLQC), 50 ng/mL (low quality control, LQC), 500 ng/mL (middle quality control, MQC), and 1000 ng/mL (high quality control, HQC), respectively. The IS solution (1000 ng/mL) was prepared from the intermediate solution.

All plasma samples were thawed and equilibrate at room temperature before analysis, and then centrifuged at 10000 ×g for 10 min at 4°C. 5 μL ZgI standard solution and 10 μL IS solution were added to 45 μL of plasma sample and vortexed for 30 s. Next, 200 μL acetonitrile was added to each sample followed by vortexing for 1 min. Finally, samples were centrifuged at 10000 ×g for 10 min at 4°C. 150 μL of supernatant was transferred and 10 μL sample for LC-MS analysis.

Method validation

The specificity of the method was assessed from blank plasma, ZgI standard solution, IS, plasma samples spiked with the ZgI and IS mixture. The plasma samples were obtained after a single oral dose of ZgI.

The linearity of the method was analyzed with calibration standards at nine different concentrations. The calibration curves were constructed by plotting the peak area ratios of analyte/ARE concentrations. Curve fitting was performed by Lab Solutions LC-MS Ver.5.85 analysis. The calibration curve was accepted if the residuals had at least 75% of the calibration standards as previous described.

The intra-batch and the inter-batch accuracy and precision were calculated by analyzing four levels of QC samples (5, 50, 500 and 1000 ng/mL, respectively). Intra-batch accuracy and precision were analyzed five times with each QC level on the same day. The inter-batch accuracy and precision were determined on three different days of each QC level. Precision was expressed as percentage (%) of Relative Standard Deviation (RSD), while the accuracy was calculated by % of Relative Error (RE).

The matrix effect was assessed by comparing the mean area response of ZgI in plasma with the mean area of standard solutions in the absence of matrix at four QC levels. Three replicates for each QC level were performed.

The stability of method was calculated by triplicate assay at four conditions (stored at 25°C for 2 h, 10°C for 24 h, -20°C for three weeks, and after three freeze-thaw cycles). The samples were evaluated by comparing the measured concentrations to freshly prepared QC samples, respectively.

Pharmacokinetics study

Twelve normal rats and leukopenia rats were orally treated with 5 or 20 mg/kg ZgI (6 rats for each group). The blood samples (0.2 ml each) were collected at 0, 5, 10, 20, 40, 60 min, 1.5, 2, 4, 8, 12, 24, 48 h after oral administrated of ZgI. All blood samples were centrifuged at 3000 ×g for 10 min and the supernatants were collected, the plasmas were stored at -20°C until detection. For the detection, 200 μL acetonitrile was added into each sample and mixed liquid was vortex for 1 min, centrifuged at 10000 ×g for 5 min. The supernatants were dried in a nitrogen environment at 40°C and the residues were dissolved in 100 μL of mobile phase.

Statistical analysis

All data were reported as mean ± standard deviation. Statistical significance of the data was analyzed by one-way univariate Analysis Of Variance (ANOVA) for comparing the means of more than two independent groups. A difference at $p<0.05$ was considered to be statistically significant (as marked as *). The higher significance level was set at $p<0.01$ (as marked as **). Pharmacokinetic parameters were calculated by DAS 3.0.

Results

Establishment of leukopenia model in rats

The results of WBCs in the rats treated with 40 or 70 mg/kg CY and compared to the rats treated with normal saline (control) are shown in table 1. The data revealed that the WBCs of rats treated with CY were significantly decreased ($p<0.05$ or $p<0.01$) compared to that of the control rats, while CY 70 mg/kg has profound effect on WBCs than that of 40 mg/kg. The results indicate that the rat model of leukopenia induced by CY has been successfully established and 70 mg/kg CY is an appropriate dose to induce leukopenia in rats. Therefore, 70 mg/kg CY with i.p. twice (day 0 and 2) was selected for induction of leukopenia for the rat model, and the WBCs in serum after 12 h were obviously reduced, suggesting that the model was successful.

Determination of chromatographic and mass spectrometric conditions

In our study, a sensitive UHPLC-MS/MS method for determination of ZgI in rat plasma at an efficacy range. The products of ion mass spectra and their corresponding fragmentations of ZgI (Figure 1a) and GA (Figure 1b) are shown in figure 2. The MRM fragmentation transitions were m/z 765.45→m/z 603.50 and m/z 469.15→m/z 355.10 for ZgI and GA, respectively. The final confirmation of the chromatographic separation method for ZgI was as follows: an isocratic elution of 10% solvent B for the initial 0.5 min, followed by a linear gradient elution of 10%-98% solvent B for the next 1.5 min, holding the composition of 98% solvent B for the next 1 min followed by column equilibration to the initial conditions over 2 min (Figure 3).

Group	Numbers	WBC ($\times 10^7$) before CY treatment	WBC ($\times 10^7$) after CY treatment (12 h)
Control	6	7.92 ± 1.68	8.02 ± 1.57
CY 40 mg/kg	6	8.03 ± 1.47	3.97 ± 1.93**
CY 70 mg/kg	6	8.19 ± 1.51	1.89 ± 0.51**

Table 1: The levels of white blood cells of rats treated with or without CY.

*p<0.05, **p<0.01 vs control.

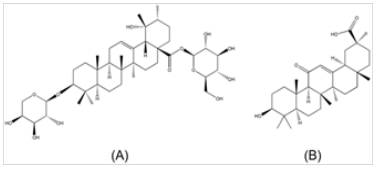

Figure 1: The chemical structures of ZgI (A) and GA (B).

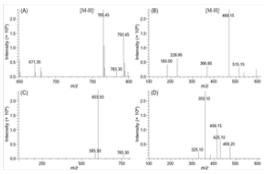

Figure 2: The MS/MS spectrums of ZgI (A) and GA (B), and the main corresponding product ion of ZgI (C) and GA (D).

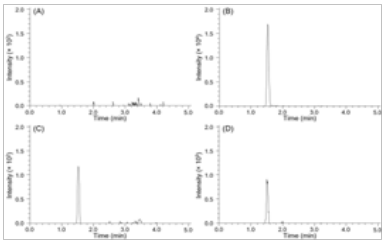

Figure 3: The MRM chromatograms of blank plasma (A), standard solution with 10 ng/mL ZgI (B), blank plasma with ZgI at LLOQ (C), and plasma of the rat with leukopenia at 20 min after a single oral administration of 5 mg/kg ZgI (D).

Method validation

The data of specificity were presented in figure 3. Blank plasma, spiked with a standard solution of 10 ng/mL ZgI, blank plasma spiked with ZgI at the Lower Limit Of Quantitation (LLOQ) level, and plasma from the rats treated with oral administration of 5 mg/kg ZgI.

It can be seen, under the established chromatographic conditions, no interference of endogenous plasma components or other impurities was observed, ZgI and IS were sufficiently separated at the retention times.

The calibration curves were linear in peak area rations over the concentration ranges of 0.5-200.0 ng/mL for ZgI. The results of linearity are shown in figure 4. After process of the calibration data and fitting the standard curve, the regression curve presented high linearity in the ranges of 0.5-200 ng/mL ZgI (R=0.9990). The linear equation was y=0.00418679x-0.000701950, where the y represents the peak area, and the x indicates the concentration ratio of ZgI and IS.

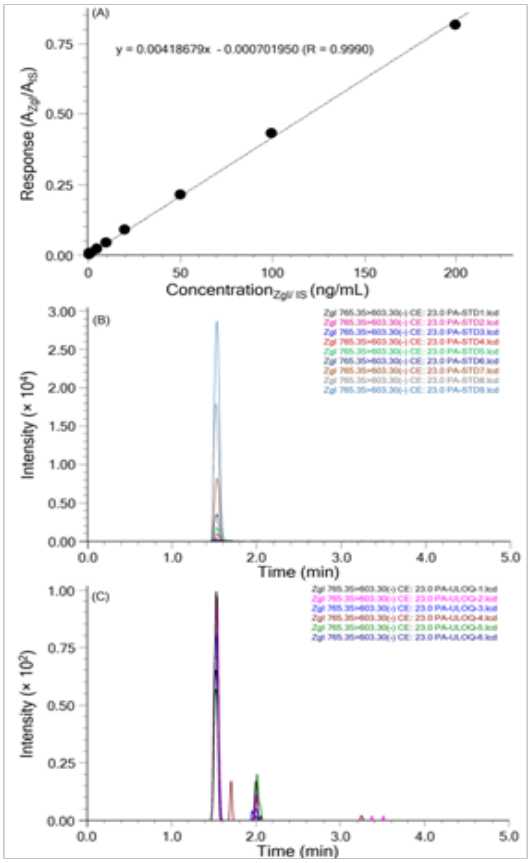

Figure 4: The linear regression calibration curves of ZgI (A), and the consecutive MRM chromatograms of ZgI with nine concentrations (B) and ZgI at LLOQ (C) (n=6).

The intra-day and inter-day precision and accuracy were performed at four QC levels and the results are shown in table 2. The data suggest that precision values at each QC level were lower than 15% and accuracy values were within 102.6%–110.8%, indicating that the assay in precision and accuracy for ZgI is in accordance with the standard of the China Food and Drug Administration (CFDA).

The matrix effect of measured compounds in plasma was defined as the overall effect of all components in the samples other than only the analytes of interest. As shown in table 3, the values of matrix effects for all levels were over 85%, indicating that the matrix effect could be ignored and free of endogenous interference in this method.

QC level	Intra-batch (%)		Inter-batch (%)	
	RSD	Accuracy	RSD	Accuracy
LLQC	13.8	104.0 ± 14.4	10.7	108.7 ± 11.6
LQC	5.7	110.8 ± 6.3	6.0	105.0 ± 6.3
MQC	1.9	105.3 ± 2.0	5.0	105.2 ± 5.3
ULQC	1.0	102.6 ± 1.0	2.2	97.0 ± 2.1

Table 2: The precision and accuracy for intra-batch and inter-batch of ZgI (n=6).

QC level	Concentration (ng/mL)	Matrix effect (%)
LLQC	0.5	98.1 ± 9.0
LQC	5	106.1 ± 6.9
MQC	50	100.3 ± 3.3
HQC	100	98.7 ± 1.8

Table 3: Matrix effect for ZgI (n=6).

The data of stability of ZgI in rat plasma under the various conditions described above are listed in table 4. The results show that the levels of RSD% of two concentrations were within 15% under the different conditions, which is within the standard of the CFDA.

Storage condition	RSD (%)	
	1 ng/mL	200 ng/mL
Short-term (25°C, 2 h)	11.9	4.3
Post-preparative (10°C, 24 h)	6.9	3.9
Long-term (-20°C, 21 days)	8.1	2.7
After three freeze-thaw cycles	6.2	3.6

Table 4: The stability of ZgI (n = 6).

Pharmacokinetics study

The profiles of mean plasma concentration-time are depicted in figure 5 and the main pharmacokinetic parameters (C_{max}, T_{max}, AUC_{0-t}, $t_{1/2\alpha}$ and $t_{1/2\beta}$) for ZgI are presented in table 5. According to the results of DAS 3.0, we find that the characteristic of pharmacokinetics of oral ZgI in rats conformed to two compartment model. Results show that C_{max}, AUC_{0-t} of ZgI are increased depend on its dosage rise, yet there are no obviously difference in other index between 5 and 20 mg/kg control rats. In contrary, all the performance of ZgI is the similar in two leukopenia rats ($p>0.05$). Compared to that of normal rats, T_{max} was significantly shortened (0.67 h to 0.33 h) and C_{max} of 20 mg/kg ZgI was remarkably decreased (7.96 ng/L to 3.40 ng/L), while elimination half-life ($T_{1/2\beta}$) was obviously prolonged (5.02 h to 18.51 h) in leukopenia rats.

Discussion

In order to make a copy of leucopenia model induced by chemotherapy, we choose anticancer drug CY to make leucopenia model due to its effect on bone marrow inhibition [20]. To establish the rat model of leukopenia, we treated the rats with CY at 40, 70, and 100 mg/kg by i.p. on the first and third days. The dose 100 mg/kg of CY was lethal to the treated rats so we could not count the WBCs for the group.

Parameters	Normal rats		Leukopenia rats	
	5 mg/kg	20 mg/kg	5 mg/kg	20 mg/kg
C_{max} (ng/L)	2.26 ± 0.74	7.96 ± 2.68	2.70 ± 0.87	3.40 ±1.26 **
T_{max} (h)	0.67 ± 0.00	0.93 ± 0.15	0.29 ± 0.08 ##	0.33 ± 0.37 **
AUC_{0-t} (ng/h/L)	29.16 ± 15.88	69.86 ±34.12	39.76 ± 17.30	57.47 ± 26.39
$AUC_{0-\infty}$ (ng/h/L)	32.80 ± 16.54	72.02 ±33.01	47.96 ± 13.39	65.34 ± 29.51
$t_{1/2\alpha}$ (h)	0.31 ± 0.04	1.22 ±0.91	0.25 ± 0.13	1.01 ± 0.85
t1/2β (h)	8.64 ± 3.65	5.02 ± 6.74	16.31 ± 6.96 ##	18.51 ± 8.41 **

Table 5: Pharmacokinetic parameters of ZgI in normal and leukopenia rats (n=6).

**p<0.01 vs normal rats treated with 20 mg/kg ZgI.
##p<0.01 vs normal rats treated with 5 mg/kg ZgI.

Chromatographic separation conditions, in particular the composition of mobile phase, play a crucial role for achieving good chromatographic behavior and proper ionization [21]. Through the investigation of the mobile phase system, the addition of 0.05% ammonia to the aqueous phase was able to significantly increase in the sensitivity and peak symmetry. In addition, acetonitrile as organic phase can provide better peak shape and lower background noise compared to methanol. Finally, we got an ideal separation with acetonitrile and H_2O both containing 0.05% ammonia as the mobile phase within 5 min. additionally, compared to methanol, protein precipitation with acetonitrile in the current experiment was more complete and less inference.

It is also necessary to have a proper IS to obtain desirable assay for mass spectrometer detection. Next, we selected optimal IS for ZgI. ZgI belongs to triterpenoid saponins and has similar structures with many other saponins including ginsenosides, ursolic acid, glycyrrhizic acid, and glycyrrhetinic acid [22]. Initially, we have tested ursolic acid, glycyrrhizic acid, and glycyrrhetinic acid as the candidates of IS. Finally, glycyrrhetinic acid was selected as the IS of ZgI because it not only belongs to the ursane type pentacyclic triterpenoid saponins but also has minimal endogenous interferences for mass spectrometer assay.

Leukopenia caused by myelosuppression, induced by Chemotherapy, is the most common side reaction in clinical treatment of tumors [23]. Leukopenia not only affects the continuation of chemotherapy, but also causes serious medical infections that lead to additional medical care or even early death [24]. Effective and efficient improvement of white blood cell levels in chemotherapy patients is a major problem which has long plagued the clinicians [25]. In this situation, Study on drug pharmacokinetics in a disease state is more important than the normal condition and more able to guide clinical medication plan [16]. The previous pharmacokinetic studies of ZgI were limited to normal animal models [9]. In present study, we revealed that the C_{max} of ZgI didn't rise with its increasing dosage by one-time administration in the state of leukopenia, which meant you may need to divide the previous dose into multiple doses to maintain its effective concentration *in vivo*, and this would be a big impact on the way of its administered, or even other relative drugs, to treat leucopenia. Contrarily, our results also showed that the metabolism time of ZgI in normal rats were almost 2 to 3 times to that of leukopenia rats, indicating that it is necessary to reduce the frequency of drug administration to prevent other side effects caused by excessive drug dose in the adjuvant chemotherapy treatment. Our study may provide a useful reference for the administration of ZGI and other related drugs in the treatment of chemotherapy-induced leukopenia.

It is no doubt that chemotherapy leads to damage of liver and change its related metabolic enzymes, as well as it induces leucopenia [26]. We should have explored the mechanism of ZgI absorption reduction caused by leucopenia, limited by the experimental conditions and other uncontrollable factors. Our current study just regrettably illuminated the pharmacokinetic features of the speed and grade of absorption of ZgI could be changed in leucopenia state. Next, we would further investigate the mechanism of ZgI absorption reduction caused by leucopenia and the distinction in pharmacokinetics of ZgI among several of species or relative diseases, and then explore the relationship between its pharmacokinetics and pharmacodynamics. Thus, providing a better and more rigorous guidance on clinical of drugs which use ZgI as the main ingredient.

Conclusion

In this study, we first compared the pharmacokinetic characteristics difference of ZgI between normal and leucopenia rats within the scope of the efficacy. The above results indicate that leucopenia state could change the pharmacokinetic characteristics of ZgI by blockingits C_{max} and slowing down its $T_{1/2\beta}$. Its exact mechanism remains to be further studied. This may provide guidance for clinical development of ZgI as an agent for the treatment of leukopenia.

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Conflicts of Interest

The authors declare no conflict of interest.

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