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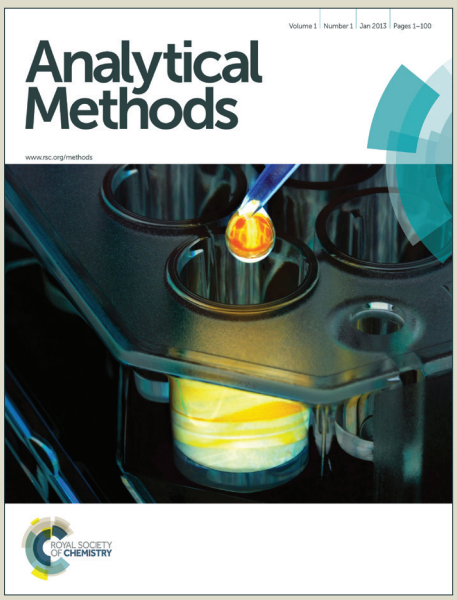

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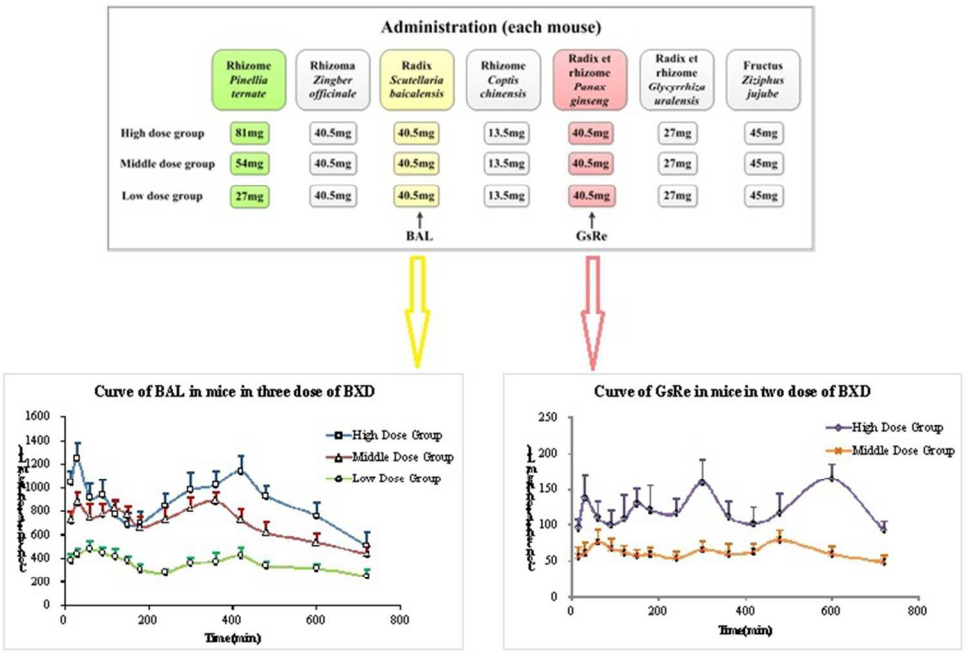
Pharmacokinetics profile of baicalin and ginsenoside Re in blood samples obtained from mice following the oral administration of three dosage groups.



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Determination of baicalin and ginsenoside Re in Banxia-Xiexin decoction using pharmacokinetics and icELISA analysis in mice

Effects of Interaction between Prescription Herbs on the Pharmacokinetics of Compounds

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Abbreviations

TCM, traditional Chinese medicine; BXD, Banxia-Xiexin decoction; BAL, baicalin; GsRe, ginsenoside Re; icELISA, indirect competitive enzyme-linked immunosorbent assay; MAb, monoclonal antibody

Abstract

Currently, little is known about how the dosage of one component of traditional Chinese medicine (TCM) alters the pharmacokinetic properties of the others in prescriptions. Banxia-Xiexin decoction (BXD) consists of concentrated granules of seven Chinese herbs. The aim of this study was to explore how dosage variations of *Pinellia ternate* (Thunb.) Breit., the monarch herb in BXD, affected the pharmacokinetics of baicalin (BAL) and ginsenoside Re (GsRe) in blood of treated mice. The decoctions containing low dose, middle dose, and high dose of *Pinellia ternate* were administered orally to mice, and indirect competitive enzyme-linked immunosorbent assays (icELISAs) using anti-BAL-monoclonal antibody (MAb) and anti-GsRe-MAb were performed to determine the concentrations of BAL and GsRe in the blood of treated mice. Results showed that variations of *Pinellia ternate* doses altered the contents of BAL and GsRe in mouse blood. In all groups, the BAL concentrations peaked at approximately 30 min and again at approximately 400 min, while GsRe showed more concentration peaks than BAL. The AUC_{0-t} of BAL showed a linear relationship with the dose of *Pinellia ternate,* and the AUC_{0-t} of GsRe also showed a direct relationship with the dose of *Pinellia ternate*. IcELISA may represent a feasible method for the study of the pharmacokinetics of TCM. Moreover, these results provide a meaningful basis for evaluation of the interactions between components in a complex prescription on their pharmacokinetics.

Keywords

Metabolic Interaction; Pharmacokinetics; Banxia-Xiexin decoction; IcELISA; Baicalin; Ginsenoside Re

Introduction

Banxia-Xiexin decoction (BXD) consists of rhizome *Pinellia ternate* (Thunb.) Breit., rhizome *Zingber officinale* Rosc., radix *Scutellaria baicalensis* Georgi., rhizome *Coptis chinensis* Franch., radix et rhizome *Panax ginseng* C.A. Mey., radix et rhizome *Glycyrrhiza uralensis* Fisch., and fructus *Ziziphus jujube* Mill. Of the seven herbs, rhizome *Pinellia ternate* (Thunb.) Breit. is considered to be the monarch herb in BXD, and compared to other herbs in this prescription, *Pinellia ternate* plays the most important roles. As the monarch herb, changes in either the quality or quantity of *Pinellia ternate* may have effects on other components in the prescription. Thus, the monarch herb is a key element of traditional Chinese medicine (TCM) compatibility. For this reason, *Pinellia ternate* was chosen as a variable and its effects on other components in BXD were studied. Although *Pinellia ternate* is the monarch herb in BXD, the other components also have important medicinal properties. Baicalin (BAL), the main constituent of radix *Scutellaria baicalensis* Georgi., is considered as the index component [1]. In addition, Ginsenoside Re (GsRe) is an important component in radix et rhizome *Panax ginseng* C.A. Mey. [2].

A major feature of TCM is the compatibility among prescriptions, which functions to treat various diseases through the potential interactions between the herbs to maximize the efficacy and minimize the adverse effects [3]. However, currently it is unclear whether the medicine is effective for disease treatment due to only the herbal combination or also depending on the dose of the herbs present in the formulation. Variations in the

herb dosage of the prescription may affect the pharmacology of the TCM, so the significance of the herb dosage on the therapeutic effects cannot be ignored. The dose-effect relationship of the components in prescriptions is one of the key problems in the theory of TCM.

The metabolism of each particular component of the formulation is considered to be essential for the compound compatibility [4]. The pharmacokinetic parameters may reflect the mechanism of compatibility in different prescriptions, and changes in dose or herbs may significantly alter the pharmacokinetics. For example, a recent study of drug compatibility demonstrated that the combination of Radix Aconiti Laterlis with Radix Glycyrrhizae resulted in significant changes in the pharmacokinetics of aconitine, mesaconitine and hypaconitine detected in rat plasma [5]. Further, co-administration of danshensu altered the pharmacokinetic effects and tissue distribution of paeonol in rats, particularly in heart and brain [6]. In addition to metabolism, the combination and dose of herbs may alter absorption of the medicine. In the Shaoyao-Gancao decoction, increased absorption of some of the Shaoyao components may be related to a reduction in the absorption of some Gancao components [7].

Currently, besides high-performance liquid chromatography (HPLC), numerous bioanalytical methods have been developed and applied in pharmacokinetic studies *in vivo*, such as liquid chromatography-mass spectrometry (LC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and ultra-fast liquid chromatography-tandem mass spectrometry (UFLC–MS/MS). The feasibility of these methods have been validated, [8-11] despite the fact that they require relatively large

amounts of samples for testing and complex pre-treatment methods for detection. In this study, a more practical approach, indirect competitive enzyme-linked immunosorbent assay (icELISA) was applied, which is based on small molecule monoclonal antibodies and has high sensitivity and specificity [4]. This technique can be used for the durative analysis of small amounts (~5 μL) of blood samples obtained from animals.

This method was used to detect the concentrations of BAL and GsRe in the blood of mice following administration of BXD with different doses of *Pinellia ternate*, in order to study the changes in the pharmacokinetics of BAL and GsRe in BXD.

Experimental

Materials

The following concentrated granules of Chinese herbs were purchased from China Resources Sanjiu Medical & Pharmaceutical Co., Ltd. (Beijing, China): Rhizome *Pinellia ternate* (Thunb.) Breit., alumina prepared (batch number: 1209001H); rhizome *Zingber officinale* Rosc. (batch number: 1207002H); radix *Scutellaria baicalensis* Georgi. (batch number: 1212001S); rhizoma *Coptis chinensis* Franch. (batch number: 1210003S); radix et rhizome *Panax ginseng* C.A. Mey. (batch number: 1211001S); fructus *Ziziphus jujube* Mill. (batch number: 1208001H); and radix et rhizome *Glycyrrhiza uralensis* Fisch., honeyed (batch number: 1211002S).

BAL (purity: 95.2%, batch number: 110715-200815) was purchased from the National Institutes for Food and Drug Control (NIFDC, China). GsRe (purity: 99.50%,

batch number: STA-25142009) was purchased from Shanghai Standard Biotech Co. Ltd. (Shanghai, China). Flat-bottom 96-well microplates (NuncImmulon 4HBX) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Coating antigen BAL-poly-l-lysine (BAL-PLL), GsRe-PLL, anti-BAL monoclonal antibodies (MAbs) and anti-GsRe MAbs purified from ascitic fluid were produced in our laboratory [12, 13]. Goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (GaMIgG-HRP; whole molecule) was purchased from GE Healthcare (NJ, USA). Ninety-six well immunoplates were purchased from Corning Incorporated (NY, USA). Skim milk was purchased from BD Corporate (Beijing, China). 3,3,5,5-Tetramethylbenzidine (TMB) was purchased from Sigma-Aldrich, Co. (Beijing, China). All other commercial chemicals used in this study were of analytical reagent grade and were obtained from Sinopharm Chemical Reagents Beijing Co., Ltd. (Beijing, China).

Instruments

An electronic balance (BS124-S) was obtained from Sartorius (Gottingen, Germany). A spectrophotometric microtiter reader was purchased from Thermo Fisher Scientific (Multiskan MK3, NY, USA) and was used for the absorbance measurements. The electro-heating standing-temperature cultivator (DRP-9082) was purchased from Sumsung Experiment Instrument Co., Ltd. (Shanghai, China).

Buffers and Solutions

The compositions of the buffers and solutions used in this study were as follows:

phosphate-buffered saline (PBS)–NaCl (137 mmol·mL^{-1}), $Na_2HPO_4 \cdot 12H_2O$ (10 mmol·mL^{-1}), KCl (2.68 mmol·mL^{-1}), and KH_2PO_4 (1.47 mmol·mL^{-1}), pH 7.4; carbonate-buffered saline (CBS)–Na_2CO_3 (15 mmol·mL^{-1}), $NaHCO_3$ (35 mmol·mL^{-1}), pH 9.6; washing buffer, PBS with 0.05% Tween-20 (PBS-T); blocking buffer, 1% gelatine in deionised water; TMB substrate solution, combination of Part A (10 mL; 24.3 mL of 0.1 mol·mL^{-1} citric acid, 25.7 mL of 0.2 mol·mL^{-1} Na_2HPO_4, and 50 mL of deionised water), Part B (0.5 mL; 2 mol·mL^{-1} of TMB in ethanol), and Part C (32 μL of 0.75% H_2O_2); stopping solution, 2 mol·mL^{-1} H_2SO_4.

Animals and drug administration

This study was performed according to the Guidelines for the Care and Use of Laboratory Animals and was approved by the Committee of Ethics of Animal Experimentation of Beijing University of Chinese Medicine using the IRB code 2013BZHYLL00106. Twenty-five male Kunming mice, weighing 30 ± 2 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were housed in an environmentally controlled breeding room for 1 week prior to the start of the experiments and provided with standard laboratory food and water *ad libitum*. Mice were fasted overnight and additional 12 h prior to drug administration. The animals were then randomly divided into the following groups: low dose group, BXD with 1/2 of normal dose of rhizome *Pinellia ternate* (Thunb.) Breit.; middle dose group, BXD with normal dose of rhizome *Pinellia ternate* (Thunb.) Breit.; high dose group, BXD with 3/2 of normal dose of rhizome *Pinellia ternate* (Thunb.) Breit. The concentrated granules of all groups were dissolved in 70-80°C water and

cooled before administration to the mice.

The seven Chinese herbs and doses in BXD are shown in **Figure 1**. Chinese medicine dispensing granules have been incorporated into the category of TCM management since 2001 [11]. The concentrated granulates were prepared as follows: Chinese herbal pieces were obtained, decocted twice, combined with boiling liquid, filtered, and then concentrated the filtrate into paste. To obtain granules, proper amount of accessories were added, combined and then the mixture was dried and additional accessories were added, mixed and granulated. All herbs were prepared according to the Pharmacopoeia of People's Republic of China, which was formulated by the Committee of National Pharmacopoeia.

Samples and preparation

Blood samples (5 μL) were collected via tail vein with a quantitative capillary (predipped with heparin sodium) prior to drug administration and then at 15 min, 30 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 10 h and 12 h after drug administration. The blood was diluted 10-fold with PBS immediately after harvest and was stored at −20°C until further analysis.

IcELISA for the determination of BAL and GsRe

The icELISA protocol used to measure the concentrations of BAL and GsRe was previously established by our group [13, 15]. Moreover, the determination of BAL in mouse blood using icELISA has been assessed [16]. Assay validation, including evaluations of standard curves, precision, stability, accuracy, and recovery of BAL and

GsRe in the blood and PBS were also performed. The icELISA procedures were as follows: Each well of 96-well microplates was coated with 100 μL of coating antigen diluted with CBS. Plates were incubated for 1 h at 37 °C, and then washed three times with PBS-T. 200 μL of blocking buffer were added to each well, and the plates were sealed and blocked for 1 h at 37°C. 50 μL of MAbs diluted with PBS were added to each well, followed by addition of equal volume of the blood samples diluted with PBS. After incubation for 1 h at 37 °C, the plates were washed three times. Then, 100 μL of goat anti-mouse IgG diluted 1:10,000 in PBS were added to each well and the plates were sealed and incubated for 0.5 h at 37 °C. After three washes, 100 μL of TMB substrate solution were added. The plates were sealed, protected from light and incubated at 37 °C. The reaction was stopped after 15 min by adding 50 μL of stop solution and the plates were read at 450 nm. Blank blood and PBS were used as negative control and blank, respectively.

Pharmacokinetics

Pharmacokinetic parameters, such as the mean maximum blood concentration (C_{max}), time of maximum concentration (T_{max}), terminal elimination half-life, area under the curve (AUC_{0-t}), and mean residence time (MRT) were calculated using a noncompartmental model with Kinetica software version 5.0 (Thermo Fisher Scientific).

Results

The icELISA used to detect BAL and GsRe in mice blood samples was previously

used in detection of BAL. In our experiments, a linear correlation was obtained for BAL concentrations in the range from 2.67 to 1029.00 $\mu g \cdot L^{-1}$. The regression equation was Y=0.154X-0.0087 with a correlation coefficient of 0.974. The average recovery was 100.9%, and the relative standard deviation of measurements was <3% in the intra-assay and <4% in the inter-assay. When determining the GsRe concentrations, a linear relationship between the optical density and doses in the range of 7.80–500.00 $\mu g \cdot L^{-1}$ ($R^2 = 0.992$) was obtained with a detection limit of 0.39 ng and a half-maximal inhibitory concentration of 64.4 $ng \cdot mL^{-1}$. The average recovery of GsRe was 99.32%. The intra-assay relative standard deviations (RSDs) were <5.77%, while the inter-assay RSDs were <8.76%, suggesting that this assay was very accurate and stable. All the validation parameters met the acceptance criteria of the United States Food and Drug Administration (US FDA) guidance (2001).

The concentrations of BAL and GsRe were detected, and the mean concentrations were calculated and plotted. **Figure 2** and **Figure 3** reflect how the concentrations of BAL and GsRe vary versus time in different dose groups. On the basis of this assay, the C_{max}, T_{max}, AUC_{0-t} and MRT were calculated. The pharmacokinetic parameters obtained from the blood samples of mice following oral administration of BXD with different doses of *Pinellia ternate* are shown in **Table 1**.

The AUC_{0-t} of BAL calculated for the high, middle and low dose groups was 614476.20 ± 47246.14, 488089.60 ± 34938.73 and 231276.44 ± 39295.53 mg·min·L^{-1}, respectively, while the AUC_{0-t} of GsRe for the high and middle dose groups was 88340.93 ± 9419.59 and 44519.43 ± 4309.96 mg·min·L^{-1}, respectively. The differences

in the AUC_{0-t} between different dose groups for both BAL and GsRe suggest that these parameters increased with increasing doses of *Pinellia ternate*. The AUC_{0-t} of BAL after oral administration with high, middle, and low doses of *Pinellia ternate* showed a linear relationship with the dose of *Pinellia ternate* (**Figure 4**, R^2= 0.9628), and the AUC_{0-t} of GsRe also varied with the dose of *Pinellia ternate*.

Discussion

This study demonstrated the occurrence of bimodal phenomena in the mean concentration versus time for BAL quantified from the mouse blood samples from all dose groups. The maximum concentration was measured at two different time points following the first administration of BXD at approximately 30 min and the second administration at approximately 400 min. These results were consistent with previously reported data. There were no significant differences in the BAL concentrations at peak times between different groups of mice, suggesting that the administration of different doses of *Pinellia ternate* can lead to varied plasma concentrations of BAL, but has little effect on the characteristics of pharmacokinetics curves of BAL. In contrast, the GsRe concentrations in blood exhibited more peaks. Despite these inconsistencies between the BAL and GsRe concentrations, some similarities between the pharmacokinetics of BAL and GsRe were found, including the time-concentration profiles, both of which were affected by the dose of *Pinellia ternate* in BXD. As shown in Figure 4, the $AUC_{0-720min}$ of BAL showed a good dose-related increase from 0.9 to 2.7 $g \cdot kg^{-1}$ (R^2=0.9628), suggesting that *Pinellia ternate* dramatically affected the absorption of

BAL in mice. This provides a possible way in clinical medicine, which means if it is necessary to enhance the efficiency of a component, besides increasing its clinical dosage, another choice is to promote its absorption by changing the doses of other related compounds in the prescription.

Prescription is an essential approach to ensure the safety and effectiveness of clinical medicine. Due to its multi-component and multi-target characteristics, studies on prescription medicines have mostly focused on those with limited components. Thus, it requires not only TCM theory but also modern analysis to elucidate the mechanism of actions of different prescriptions. One strategy to explore the correlation between the components and pharmacological action of medicines with respect to pharmacokinetics is based on their concentration-time-effect relationships [17]. Rapidly, techniques are being developed to study the pharmacokinetics of TCM [18]. For example, the effect of non-flavonoid components on flavonoids [19], effects of *Platycodonis* in the Shengxian decoction [20], a compatibility study of the components of Zuojinwan [21], a study of the Shaofu Zhuyu decoction using ultra performance liquid chromatography (UPLC) [22], and the interaction between PUE and BAL in Gegen Qinlian decoction [23] have been investigated.

This work suggests that variations in the dose of *Pinellia ternate* in BXD can change the pharmacokinetics of BAL and GsRe, which highlights the necessity of pharmacokinetic studies in TCM. Moreover, icELISA was successfully applied to quantify the contents of the Chinese medicine components in blood samples obtained from the same mouse over a continuous duration, which provides a feasible method for

studying the pharmacokinetics of TCM.

This study demonstrated the differences in the time-concentration curves of BAL and GsRe following the oral administration of different doses of *Pinellia ternate* in BXD, suggesting that variations in compatibility may cause pharmacokinetic changes in the absorption or metabolism of other components in the prescription. This finding provides a powerful reference for clinical guidance on individual prescription and medication based on different diseases and syndromes.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81274043, 81373542), and the Classical Prescription Basic Research Team of Beijing University of Chinese Medicine.

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Table 1. Pharmacokinetic parameters of BAL and GsRe after the oral administration of different dosages of BXD in mice (mean ± SD, n = 6)

BAL	High dose group	Middle dose group	Low dose group
C_{max} ($mg \cdot L^{-1}$)	1272.28 ± 97.80	935.08 ± 74.15	519.77 ± 37.70
T_{max} (min)	96.00 ± 141.99	204.00 ± 166.61	166.67 ± 176.28
AUC_{0-720} ($mg \cdot min \cdot L^{-1}$)	614476.20 ± 47246.14	488089.60 ± 34938.73	231276.44 ± 39295.53
AUC_{tot}	891555.40 ± 244718.53	794419.60 ± 167474.23	505996.88 ± 400825.80
MRT (min)	623.40 ± 270.61	745.16 ± 215.71	994.48 ± 1064.92

GsRe	High dose group	Middle dose group
C_{max} ($mg \cdot L^{-1}$)	184.17 ± 7.64	88.53 ± 12.60
T_{max} (min)	423.00 ± 203.20	300.00 ± 191.83
AUC_{0-720} ($mg \cdot min \cdot L^{-1}$)	88340.93 ± 9419.59	44519.43 ± 4309.96
AUC_{tot}	162552.25 ± 30980.03	89681.61 ± 56715.43
MRT (min)	849.77 ± 316.19	1020.59 ± 969.70

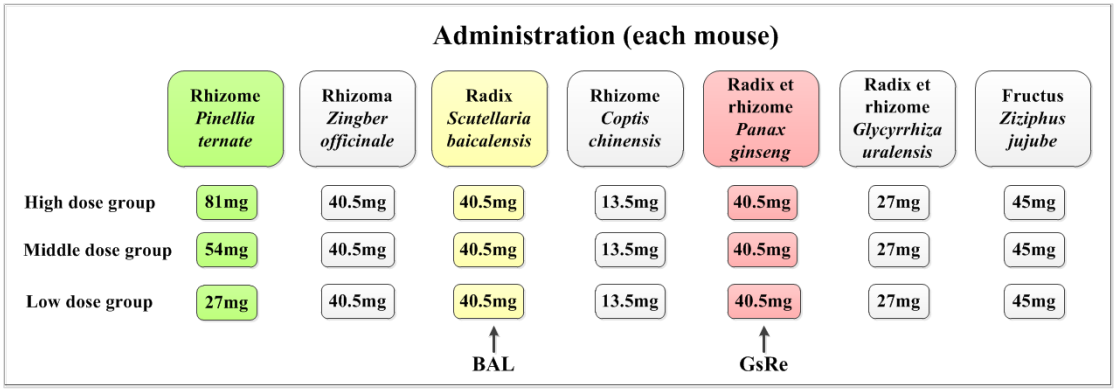

Fig. 1 Dosage administration of each mouse in the three groups.

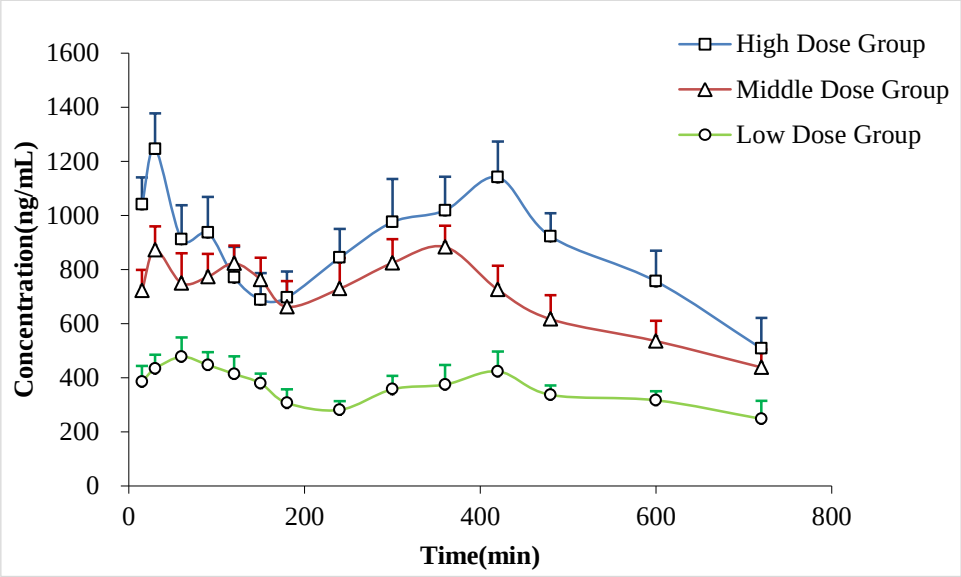

Fig. 2 Comparison of the mean BAL concentration-time curves in blood samples obtained from mice following the oral administration of three dosage groups. The decreased C_{max} values of BAL were nearly multiplied in the high dose group to the low dose group.

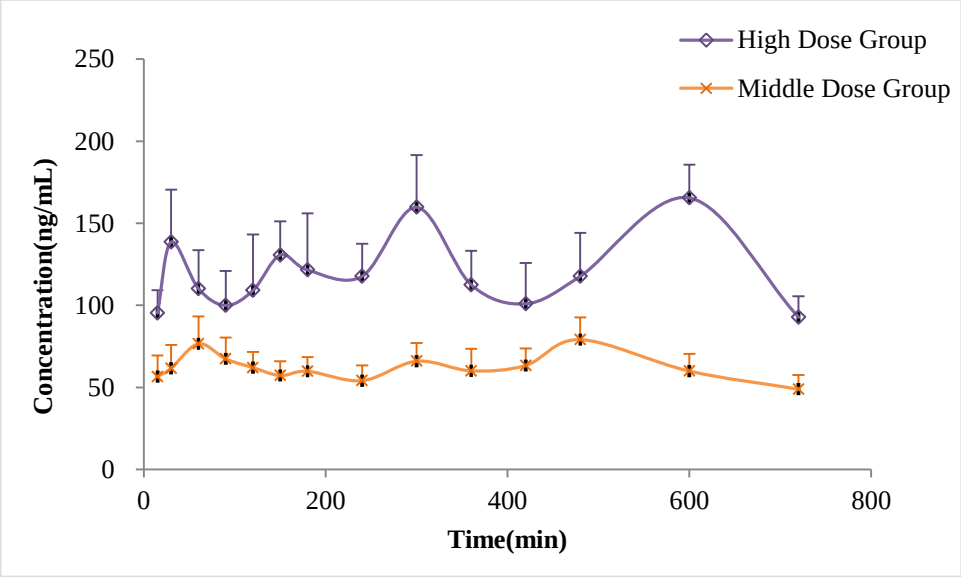

Fig. 3 Comparison of the mean GsRe concentration-time curves in blood samples obtained from mice following the oral administration of two dosage groups.

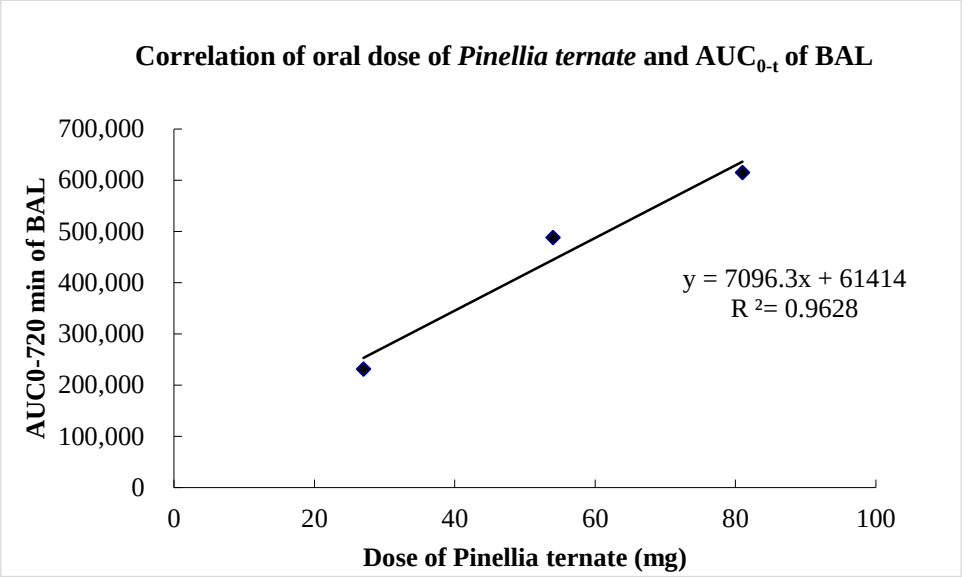

Fig. 4 Correlation of oral dose of *Pinellia ternate* and AUC_{0-t} of BAL. The X-axis represents the dosage of *Pinellia ternate* administrated to each mouse; Y-axis reflects the AUC of BAL, according to results in Table 1. It showed a good correlation between the AUC of BAL and dose increase of *Pinellia ternate* (R^2=0.9628).