Pharmacokinetic–pharmacodynamic modeling to study the anti-dysmenorrhea effect of Guizhi Fuling capsule on primary dysmenorrhea rats

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**Keywords:**
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Primary dysmenorrhea
Pharmacokinetic–pharmacodynamic model
Ratios of PGE2/PGF2α and 6-Keto-PGF1α/TXB2

**Abstract**

**Background:** Primary dysmenorrhea (PDM) is one of the most common gynaecological disorders among women, which seriously affects women's life quality due to its high incidence rate. Guizhi Fuling capsule (GZFLC), a well-known traditional Chinese medical prescription, has been widely used to treat gynaecological blood stasis syndromes such as PDM. However, its mechanisms of action and combination were still unknown.

**Purpose:** The aim of this study was to develop a pharmacokinetic–pharmacodynamic (PK-PD) model to assess time-concentration-effect relationships for anti-dysmenorrhea effect of GZFLC and provide better understanding for mechanisms of action and combination of GZFLC.

**Study design and methods:** The PDM rats model was induced by oxytocin exposure following estradiol benzoate pretreatment. Gallic acid (GA), amygdalin (AMY), albisflor (ALB), prunasin (PA) and cinnamic acid (CA) were evaluated as bioactive ingredients for investigating PK processes. GA, AMY, ALB and PA exhibited appropriate PK parameters and were selected as the PK markers to map the anti-dysmenorrhea effect of GZFLC. A PK-PD model was established on the basis of GA, AMY, ALB and PA plasma concentrations vs. the values of two ratios (PGE2/PGF2α and 6-Keto-PGF1α/TXB2), by a two-compartment PK model with a simple Emax model to explain the time delay between the drug plasma concentrations of PK markers and the anti-dysmenorrhea effect.

**Results:** The PDM rat model has been successfully established. Compared with the normal treated group, the bioactive ingredients in PDM treated group exhibited significant changing trends of PK behaviors, such as better absorption and distribution, slower elimination and delays in reaching the maximum concentration (Tmax). The analysis of PK-PD parameters indicated that the active metabolites and prototypes of bioactive ingredients in GZFLC were inclined to regulate the activity of prostacyclin synthetase and thromboxane synthetase to control the production of TXA2 and PGI2 so as to treat PDM. As the main effective medicinal materials for the treatment of PDM in GZFLC prescription Persicae Semen, Moutan Cortex and Paonia lactiflora Pall, Persicae Semen played the most important role, while the role of Paonia lactiflora Pall was the weakest.

**Conclusion:** The PK-PD model results provided scientific clarification for combining mechanisms of GZFLC prescription and a better understanding for biosynthetic mechanisms of four prostaglandins (PGE2, PGF2α, 6-Keto-PGF1α and TXB2) in the treatment of PDM by GZFLC. Investigations on the relationship between the effects and the bioactive ingredients are of benefit to explore the mechanisms of action and combination for traditional Chinese medical prescriptions (TCP) and facilitate the development of future clinical applications of TCP.

**Abbreviations:**
ALB, albisflor; AMY, amygdalin; ARA, arachidonic acid; AUCA0-t, area under the time-concentration curve from zero to a definite time t; CA, cinnamic acid; Cmax, the peak plasma drug concentration; CL/F, clearance rate; COX, cyclooxygenase; Emax, maximal effect; GA, gallic acid; PTG, primary dysmenorrhea treated group; GZFLC, Guizhi Fuling capsule; IS, internal standard; LLOQ, lower limit of quantification; MRT0-t, mean residence time from zero to a definite time t; NTG, normal treated group; NSAIDs, nonsteroidal anti-inflammatory drugs; OCS, oral contraceptives; PA, prunasin; PDG, primary dysmenorrhea model group; PDM, primary dysmenorrhea; PG12, prostaglandin E2; PGF2α, prostaglandin F2α; PGs, prostaglandins; PK-PD, pharmacokinetic–pharmacodynamic; QC, quality-control; TCM, Traditional Chinese medicines; TCP, traditional Chinese medical prescriptions; Tmax, time to peak plasma drug concentration; TXB2, thromboxane B2; t1/2, half-life of elimination; V/F, apparent volume of distribution; β-EP, β-endorphin; 6-Keto-PGF1α, 6-Keto-Prostaglandin F1α

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Introduction

PDM is one of the most common gynaecological disorders among adolescent girls and refers to the recurrent and crampy pain occurring at the lower abdomen and waist with no organic disease (Chen et al., 2014; Ryan, 2017). PDM results from abnormal and enhanced uterine contractions and is closely related to psychological factors (Gagau et al., 2013). The pain often occurs at start of menses, lasts 1-3 d and accompanies by systemic symptoms (Ryan, 2017). According to the epidemiological studies, the prevalence varies of PDM in female adolescents is 20% to 90%, and 15% of reproductive women suffer from severe PDM, which seriously affects women’s life quality (Kazama et al., 2015; Nguyen et al., 2015).

Some studies have shown that the aetiology of PDM is due to increased abnormal uterine contraction caused by excessive secretion of uterine prostaglandins (PGs) (Pan et al., 2014). The four principal PGs, prostaglandin F2α (PGF2α), prostaglandin E2 (PGE2), 6-Keto-Prostaglandin F1α (6-Keto-PGF1α, the stable metabolite of prostacyclin) and thromboxane B2 (TXB2, the metabolete of TXA2), were produced through the arachidonic acid (ARA) under the action of cyclooxygenase (COX, Fig. S2), which have been proved to be the major factors in the pathogenesis of PDM (Dawood and Khan-Dawood, 2007b; Hayes and Rock, 2002). PGF2α and TXA2 are strong stimulants of uterine contractions and vasoconstrictor, respectively (Shi et al., 2011; Yang et al., 2015). Therefore, PGF2α and TXA2 will not only increase the tension in the uterus and reduce blood flow but also promote thrombosis. However, the other two PGs, PGE2 relax the uterus by inhibiting contractions and spontaneous activity of smooth muscle. Prostacyclin (PGI2) relieves PDM symptoms by dilating blood vessels, preventing platelet aggregation and thrombosis (Cella et al., 2006; Dawood and Khan-Dawood, 2007a; Hertelendy and Zakar, 2004; Shi et al., 2011; Siemieniuch et al., 2009). Clinical studies have shown that PGF2α and TXB2 levels of PDM patients were much higher than those of asymptomatic normal people (Dawood and Khan-Dawood, 2007b), while 6-Keto-PGF1α and PGE2 levels of PDM patients were opposite (Shi et al., 2011; Yang et al., 2015). Therefore, the increased level of PGE2/PGF2α and 6-Keto-PGF1α/TXB2 have been considered as the indicators of clinical efficacy for PDM (Shi et al., 2011). In addition, modern studies suggested that β-endorphin (β-EP) is a neuroendocrine hormone and targeting uterus with endogenous analgesic effects (Jia et al., 2006).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most common pharmacological treatments for PDM (Marjoribanks et al., 2009). Moreover, PDM has also been proved to be associated with sex hormones and ovarian steroid related diseases. Therefore, the oral contraceptives (OCS) are also applied to treat PDM (Ostad et al., 2001). Although the efficacy of NSAIDs and OCS is rapid and remarkable, there are many side effects on the liver, kidney, gastrointestinal function and even cardiac systems (Zahradnik and Groth, 2010). Due to these disadvantages of NSAIDs and OCS, traditional Chinese medicines (TCM) are considered as the feasible alternative to treat PDM. TCP are primary means of clinical applications of TCM. Typical prescriptions for treating PDM are Xiang-Fu-Si-Wu Decoction, Gegen Decoction, Dang-Gui-Shao-Yao-San, Guizhi Fuling Wan and so on, which have been proved to be safer and more effective with less side effects in the treatment of PDM and exert synergistic therapeutic efficacies through multiple TCM (Chen et al., 2014; Liu et al., 2013; Yang et al., 2016).

GZFLC is one of the most widely used TCP for the treatment of PDM, which was originated from Guizhi Fuling Wan, first described in the Essential Prescriptions from the Golden Cabinet (Jin Kui Yao Lue) compiled by Zhang Zhongjing (150-219 AD), a prestigious Chinese physician in the Eastern Han Dynasty. The prescription consists of five medicinal materials, including Cinnamomum cassia (L.) J.Presl (Cassia bark), Poria cocos (dried sclerotium of the fungus (Schw.) Wolf (Poria), Paeonia lactiflora Pall (the dried root of Herbaceous peony), Moutan Cortex (dried root bark of Paeonia suffruticosa Andrews) and Pervicie Semen (dried ripe seed of Amygdalus persica L.) with the same proportions, and was widely used to treat gynecological blood stasis syndromes such as PDM, endometriosis, hysteromyoma and so on (Hu et al., 2014; Sun et al., 2016). In addition, GZFLC has already finished the Phase II clinical trial for treatment of PDM by the USA Food and Drug Administration (NCT01588236). However, the mechanisms of action and combination of GZFLC for treatment of PDM are still unknown.

Pharmacokinetic–pharmacodynamic (PK-PD) modeling in drug research is an available approach to characterize the interrelated dynamic connection between vivo process and efficiency of drugs, which is able to provide the scientific basis for the effective substance basis and revealing the therapeutic mechanism of TCM, has important significance for the realization of modernization and internationalization of TCM (Song et al., 2013; Zhang et al., 2016; Zhou et al., 2016). Moreover, PK-PD modeling has been widely used in pre-clinical in vivo studies, which contributes to a comprehensive and accurate understanding of the efficiency process that changes with time and plasma concentration, thereby provides valuable references for improving the curative efficacy, optimizing the clinical dosage and reducing the toxic and side effects (Danhof et al., 2008; Penney and Agoram, 2014).

In animal experiments, estradiol benzoate and oxytocin are used to induce uterine contractions and PDM model (Huang et al., 2016; Liu et al., 2011). In this paper, PK-PD modeling was first applied to the treatment of PDM with GZFLC. Gallic acid (GA, from Paeonia lactiflora Pall and Moutan Cortex), amygdalin (AMY, from Pervicie Semen), albitiflorin (ALB, from Paeonia lactiflora Pall), prunasin (PA, from Pervicie Semen) and cinnamic acid (CA, from Cinnamomum cassia) are main index bioactive ingredients in GZFLC and chosen as PK markers candidates to make a correlation with the anti-dysmenorrhea effect of GZFLC by PK-PD modeling (Zhao et al., 2015). The levels of two ratios (PGE2/PGF2α and 6-Keto-PGF1α/TXB2) in plasma are chosen as PD markers which were obtained from the same individual as in pharmacokinetics. The findings obtained from the PK-PD model results are expected to provide scientific basis for clarifying mechanisms of action and combination of GZFLC in the treatment of PDM.

Materials and method

Chemicals and reagents

GZFLC were provided by Jiangsu Kanion Pharmaceutical Co. Ltd (Jiangsu, China, No. 20160224). Reference standards of CA, GA, AMY and the internal standard (IS) bendrofluamide were all obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Reference standards of PA and ALB were purchased from Chengdu Chroma-Biotechnology Co. Ltd (Sichuan, China, No. CHB150827, CHB150714). The purity of each reference standard was more than 99.0%. Estradiol benzoate injection and oxytocin injection were purchased from Ningbo second hormone Products (Beijing, China). Reference standards of PA and ALB were provided by Jiangsu Kanion Pharmaceutical Co. Ltd (Jiangsu, China, No. 20160804 and No. 20160516). Aspirin enteric-coated tablets were obtained from Bayer HealthCare Manufacturing S.r.l (No. 20160906). PGE2, β-EP, PGF2α, TXB2 and 6-Keto-PGF1α ELISA kits were supplied from Shanghai Enzyme-linked Biotechnology Co., Ltd (Shanghai China). Methanol and acetonitrile (HPLC grade) were supplied by Fisher Scientific (Pittsburgh, PA, USA). Formic acid (HPLC grade) was purchased from Dikma (Richmond Hill, NY, USA). All other reagents were analytical grade. Distilled water was provided by Wahaha Co. Ltd. (Hangzhou, China).

Animals

Fifty female Sprague-Dawley (SD) rats (250–280 g) were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University. The SD rats were housed and handled in laboratory conditions and acclimatized to five days. Animal study was carried out following the regulations for animal experimentation issued by the State...
Committee of Science and Technology of the People's Republic of China.

Primary dysmenorrhea rats model preparation

Thirty-two female SD rats were evenly divided into four groups as follows: normal control group (NCG), model control group (MCG), GZFLC group and positive group (PG). Except the NCG (subcutaneous injection of saline), estradiol benzoate and oxytocin were used to induce PDM rats model in other groups. Estradiol benzoate (0.35 mg/kg) was administrated by subcutaneous injection for seven consecutive days. The GZFLC group, MCG and PG rats were orally administrated with GZFLC (2.76 g/kg), distilled water of the same volume and aspirin (1.8 g/kg) for eight days in the modeling period, respectively. On the eighth day, oxytocin (15 IU/kg) was administrated by intraperitoneal injection 2 h after the last administration. The number of writhing was calculated within 1 h after oxytocin injection. All of the rats were then sacrificed and the uterine and ovarian tissues were collected for further analysis.

Biochemical analysis in uterine, ovarian tissue and plasma

The levels of the PGF\textsubscript{2\alpha} and PGE\textsubscript{2} in the uterine, ovarian tissue and the levels of β-EP in rats plasma were determined by the corresponding kits.

Examination of histopathology

Uterine tissues excised from each rat were fixed in 10% formalin/saline and stained with hematoxylin and eosin (H&E). Histopathological changes of uterine tissues and capturing of images were carried out using a light microscope (OLYMPUS, DP72, Japan).

UPLC-MS/MS instruments and conditions

The therapeutic efficacity of GZFLC on PDM was evaluated by the levels of PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, 6-Keto-PGF\textsubscript{1α}, and TXB\textsubscript{2} in PDG, NTG and PTG rats PK plasma. PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, 6-Keto-PGF\textsubscript{1α}, and TXB\textsubscript{2} concentrations were measured in strict accordance with the ELISA kit instructions.

Preparation of calibration standards and quality-control (QC) samples

For the PK investigation, an aliquot of 100 µl plasma sample was extracted with 400 µl acetonitrile after addition of 50 µl IS working solution and 50 µl 0.1% formic acid-water solution. The mixture was vortex shaken for 60 s and then centrifuged at 6,000 rpm for 10 min, the supernatant was quantitatively transferred to another new tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dried residue was reconstituted in 100 µl of initial mobile phase (5% acetonitrile and 95% water), and then centrifuged at 6,000 rpm for 10 min. 20 µl aliquot of supernatant was injected into the UPLC-MS/MS system.

Method validation

The method was validated according to the currently accepted US-FDA and European Medicines Agency Guideline on Bioanalytical Method validation (U.S., 2001) and regulation Guidance with respect to selectivity (Cigno, 2013), linearity and lower limit of quantification (LLOQ), precision and accuracy, recovery, matrix effect and stability in plasma.

Pharmacokinetic study

After verification of PDM rats model, eighteen female SD rats were divided into three groups (n = 6 per group). Rats in PDM model group (PDG) were modeled according to previously validated modeling methods, and not given to GZFLC. The GZFLC was suspended in 0.5% carboxymethyl cellulose sodium (CMC-Na) (w/v). Rats in normal treated group (NTG) received an intragastric administration of 4.0 g/kg GZFLC (equivalent to 23.72 mg/kg for GA, 48.44 mg/kg for AMY, 24.56 mg/kg for ALB and 9.56 mg/kg for PA, 4.12 mg/kg for CA). In PDM treated group (PTG), rats were induced PDM rats model in the same way and administrated with the same dose GZFLC as the NTG (4.0 g/kg) intragastrically. In the PDG, NTG and PTG, blood samples (about 0.3 ml) were obtained from the postorbital venous plexus in heparinized tubes before the dose (0 h) and at 0.083, 0.167, 0.25, 0.75, 1.5, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h after administration, which were immediately centrifuged at 6,000 rpm for 5 min to obtain plasma. Blood samples of PDG were collected at the same time points and centrifuged at the same speed and time. All the plasma samples were stored at −80 °C until analysis.

Pharmacodynamics study

The data of PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, 6-Keto-PGF\textsubscript{1α}, and TXB\textsubscript{2} in PDG, NTG and PTG rats PK plasma. PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, 6-Keto-PGF\textsubscript{1α}, and TXB\textsubscript{2} concentrations were measured in strict accordance with the ELISA kit instructions.

Pharmacokinetic-pharmacodynamic simulation

The data of PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, 6-Keto-PGF\textsubscript{1α}, and TXB\textsubscript{2} measured by corresponding ELISA kits in PDG, NTG and PTG rats plasma could be transformed into the ratios of PGE\textsubscript{2}/PGF\textsubscript{2\alpha} and 6-Keto-PGF\textsubscript{1α}/TXB\textsubscript{2} to facilitate the PK/PD simulation. Thus, the treating PDM effect of GZFLC could be simply evaluated by these two ratios so as to simplify the PK-PD model. The plasma concentrations of PA, GA, AMY, ALB and the ratios of PGE\textsubscript{2}/PGF\textsubscript{2\alpha} and 6-Keto-PGF\textsubscript{1α}/TXB\textsubscript{2} in PDG, NTG and PTG rats were used to establish the relationship between the pharmacokinetics and pharmacodynamics. After comparison among different PK-PD models such as the linear model and E\textsubscript{max} model, simple E\textsubscript{max} model for PK-PD analysis were selected based on best fitness of PK and PD data (Phoenix WinNonLin 6.4 software, Pharsight Corporation, USA). The relationship between the plasma concentrations of PA, GA, AMY, ALB and the ratios of PGE\textsubscript{2}/PGF\textsubscript{2\alpha} and 6-Keto-PGF\textsubscript{1α}/TXB\textsubscript{2} in PDG, NTG and
PTG rats was determined by the following equation:

\[ E = \frac{E_{\text{max}} \cdot C_e}{E_{\text{Co}} + C_e} \]

where \( E \) is the ratios of PGE2/PGF2α and 6-Keto-PGF1α/TXB2 and \( C_e \) is the concentrations of PA, GA, AMY and ALB in the effect site compartment; \( E_{\text{max}} \) is the maximal predicted effect that describes efficacy; \( E_{\text{Co}} \), which represents the potency, is the drug concentration in the effect compartment that evokes a one-half maximal effect.

Data analysis

PK parameters were processed by the two-compartmental method using the Drug and Statistics 2.0 software package supplied by Chinese Pharmacological Society. Time course of PA, GA, AMY and ALB in blood compartment and effect site compartment were analyzed with PK-PD models (Sheiner et al., 1979). All values were expressed as mean ± standard deviation (mean ± SD). Statistical levels were calculated using independent samples t-test (SPSS 19.0). A p value of less than 0.05 was considered statistically significantly different.

Results

Primary dysmenorrhea rats model verification

As shown in Fig. 2A and Table S2, oxytocin induced writhing in MCG, GZFLC group and PG rats. Compared with the MCG, the writhing times were significantly inhibited by the positive drugs (aspirin) and GZFLC (p < 0.01). The number of writhing in GZFLC group and PG rats were 13.6 ± 3.8 and 11.4 ± 3.3, and significantly lower than that in MCG rats (25.0 ± 8.0) (p < 0.01). Moreover, the inhibition rate of oxytocin-induced writhing [(MCG writhing times − GZFLC or PG writhing times)/MCG writhing times] in GZFLC group and PG were 45.6% and 54.4%, respectively (Table S2). In this study, it could be demonstrated that GZFLC significantly reduced oxytocin-induced writhing times.

The levels of PGE2 in the uterine, ovarian tissue and the levels of β-EP in rats plasma of the MCG decreased significantly compared with the NCG (p < 0.001) (Table S3). However, the levels of PGE2 and β-EP increased significantly in the GZFLC group and PG (p < 0.001) (Table S3 and Fig. 2B, 2C). The level of PGF2α increased significantly in the MCG compared with the NCG and decreased significantly in the GZFLC group and PG compared with the MCG. (p < 0.001, Fig. 2D). The treatment of GZFLC and aspirin promoted the ratios of PGE2/PGF2α in the GZFLC group and PG (p < 0.001) (Fig. 2E and Table S3).

Histopathological examination

As shown in NCG results (Fig. 3A), it was observed that endometrial tissue lined by high columnar epithelium, dense microvilli, rich in blood vessels and glands, no inflammation or edema, spiral arteries and stromal cells had no obvious abnormalities in morphology and structure. In MCG (Fig. 3B), endometrial tissue lined by flattened epithelium, microvilli partly fell off, the lumina of spiral arteries were narrow, stromal cells showed hydropic degeneration and a small number of inflammatory cells were observed. In PG and GZFLC groups (Fig. 3C, 3D), the number of spiral arteries and inflammatory cells were significantly reduced compared with the MCG. Moreover, hydropic degeneration of stromal cells was alleviated compared with the MCG. There was no significant difference in histopathology between PG and GZFLC group.

Method validation

Selectivity was determined by analysis of blank plasma, blank plasma spiked with five analytes (at LLOQ) and IS, and rat plasma samples after administration of GZFLC. No significant interfering signals was observed at the retention time of five analytes and IS (Fig. S1). The regression equations, correlation coefficients, linear ranges and LLOQs of five analytes were shown in Table S4. All the calibration curves exhibited good linearity with correlation coefficients better than 0.9938. The intra-day and inter-day precisions of these analytes were stable under the following conditions: 12 h at room temperature, a period of 2 weeks of storage at −80 °C, 48 h in the autosampler (4 °C) and 3 freeze-thaw cycles at −80 °C (Table S5).

Pharmacokinetics analysis

The validated method was successfully applied to the pharmacokinetics study of five compounds in rat plasma after administration of...
GZFLC in PTG and NTG rats. The mean plasma concentration-time profiles \((n = 6)\) are presented in Fig. 4, the estimated PK parameters and plasma concentrations are shown in Table 1 and Table S7.

In both PTG and NTG, CA showed fastest absorption \((T_{\text{max}}\), both 0.2 h). Compared to the NTG, \(T_{\text{max}}\) and \(t_{1/2}\) from GA, AMY, ALB and PA were longer in PTG \((p < 0.001)\), which indicated that pathological status may have delayed absorption of these bioactive ingredients. Moreover, it was found that the \(AUC_{0-\infty}\), \(C_{\text{max}}\) and \(MRT_{0-\infty}\) values of GA, AMY, ALB and PA were significantly increased in PTG, which indicated that PTG rats had better absorption and slower elimination of these bioactive ingredients. To be specially mentioned, the clearance rate \((CL/F)\) and apparent volume of distribution \((Vz/F)\) of GA, ALB and PA in PTG rats were significantly lower and higher than NTG rats, respectively, indicating that there were lower elimination and better distribution of GA, ALB and PA in PTG rats. Furthermore, greater \(Vz/F\) for AMY and CA were observed in PTG rats, indicating that AMY and CA may distribute faster to the effective sites in vivo.

**Pharmacodynamic analysis**

Levels of PGE\(_2\), PGF\(_{2\alpha}\), 6-Keto-PGF\(_{1\alpha}\), TXB\(_2\) and ratios of PGE\(_2\)/PGF\(_{2\alpha}\), 6-Keto-PGF\(_{1\alpha}\)/TXB\(_2\) in PTG, NTG and PDG rats plasma were shown in Fig. 5 and Table S8, S9. After establishing the PDM rats model and administrating GZFLC, the concentrations of TXB\(_2\) and PGF\(_{2\alpha}\) in PTG were decreased by degrees, and then reached the lowest point at 4 h and kept the concentrations within a small range of fluctuations until 24 h. Significant differences occurred for the concentrations of TXB\(_2\) and PGF\(_{2\alpha}\) from 0.0 to 0.75 h between PTG and NTG. Moreover, there was significant difference between PTG and PDG for the time points after 0.17 h \((p < 0.001)\).

The variation trends in concentrations of PGE\(_2\), 6-Keto-PGF\(_{1\alpha}\) and ratios of PGE\(_2\)/PGF\(_{2\alpha}\), 6-Keto-PGF\(_{1\alpha}\)/TXB\(_2\) were the opposite of the
Pharmacokinetic and pharmacodynamic modeling

Fig. 6 showed the mean plasma concentration-time curves (red curves) of PA, GA, ALB, and AMY in PTG rats. Significant hysteresis was observed with maximal effect (Emax) achieving at 4 h while Cmax of GA, ALB and AMY reached at 2 h, indicating that there were delays in the bioactive ingredients of GZFLC arriving the effective sites. Moreover, concentration-effect curves showed counterclockwise hysteresis manners (Fig. 7), which emphasized the delays between distribution of bioactive ingredients in plasma and effective sites and indicated that the effect site compartment was separated and not in the blood compartment. In addition, there was no significant hysteresis between distribution of PA in plasma and effective sites (Fig. 6A, E and Fig. 7A, E).

As showed in Fig. 4, PK profiles of PA, GA, ALB and AMY were well fitted to the two-compartment model with delay time. Based on the two compartmental PK model, a simple Emax model with Sheiner’s method was well fitted to describe the time course of PA, GA, ALB and AMY concentrations in blood compartments and effect site compartment (Ct) (Fig. S2), which consisted of the administered compartment, peripheral compartment, central compartment (plasma) and effect site compartment (Sheiner et al., 1979) as shown in the Fig. 8.

The time courses of the concentrations of the PA, GA, AMY and ALB in each compartment were expressed by the following differential equations:

$$
\frac{dX_i}{dt} = X_i k_a + X_i k_1 - X_i k_2 - X_i k_0
$$

$$
\frac{dX_e}{dt} = X_i k_1 - X_e k_0
$$

The anti-dysmenorrhoea effect of GZFLC was directly related to the concentrations of PA, GA, ALB and AMY in the effect site compartment. Therefore, function expression of concentrations variation of four analytes in effect site compartment was:

$$
C_t = \frac{k_a k_F X_a (k_2 - k_0)}{V_1 (\alpha - \beta) (k_0 - \alpha)} e^{-\alpha t} + \frac{k_a k_F X_a (k_3 - k_0)}{V_1 (\alpha - \beta) (k_0 - \alpha) e^{-\beta t}} + \frac{k_a k_F X_a (k_2 - k_0)}{V_1 (\alpha - \beta) (k_0 - \alpha)} e^{-\beta t} + \frac{k_a k_F X_a (k_3 - k_0)}{V_1 (\alpha - \beta) (k_0 - \alpha)} e^{-\alpha t}$$

Where t represented the time after administration; Xo, X1, X2 and X3 represented the concentrations of PA, GA, ALB and AMY in each compartment; k_a represented the absorption rate constant; k_10 represented the elimination rate constant; k_0 represented the equilibration rate constant; k_12 and k_13 represented the inter-compartmental transfer rate constant; V_1 represented the distribution volume of central compartment; \(\alpha\) and \(\beta\) represented the first-order rate constants; C_t represented the concentrations of the PA, GA, ALB and AMY in effect site compartment.

The main parameters of PA, GA, ALB and AMY calculated with the PK–PD model were summarized in Table 2. The Emax of PA, GA, ALB and AMY were 1.05, 1.17, 1.08 and 1.18 in 6-Keto-PGF_1alpha/TXB_2 model results, which were equal to 88.3%, 98.4%, 90.8% and 99.2% of the maximum ratios of 6-Keto-PGF_1alpha/TXB_2 in PTG rats, respectively. Moreover, Emax of these bioactive ingredients were 1.94, 1.94, 1.97 and 2.10 in PGE_2/PGF_2alpha model results, which were equal to 79.2%, 79.2%, 80.4% and 85.7% of the maximum ratio of PGE_2/PGF_2alpha in PTG rats, indicating that these bioactive ingredients played determinant roles in
Table 1: Main pharmacokinetic parameters of five analytes in normal treated group (NTG) and PDM treated group (PTG) rats (mean ± SD; n = 6).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>GA</th>
<th>ALB</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmax (h)</td>
<td>1.4 ± 0.3</td>
<td>2.0 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>CLz/F (l/kg)</td>
<td>18.2 ± 5.1</td>
<td>55.9 ± 54.7</td>
<td>41.2 ± 19.5</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>2.6 ± 1.1</td>
<td>5.3 ± 4.9</td>
<td>3.8 ± 2.0</td>
</tr>
<tr>
<td>AUC (μg·h/ml)</td>
<td>4.0 ± 0.8</td>
<td>4.2 ± 0.8</td>
<td>4.4 ± 1.1</td>
</tr>
<tr>
<td>Cmax (μg/ml)</td>
<td>2.6 ± 1.1</td>
<td>5.3 ± 4.9</td>
<td>3.8 ± 2.0</td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>4.7 ± 2.9</td>
<td>1.4 ± 0.3</td>
<td>1.5 ± 0.0</td>
</tr>
<tr>
<td>Date represents the mean ± SD. *p &lt; 0.05, **p &lt; 0.01, ***p &lt; 0.001, PTG versus NTG rats.</td>
<td></td>
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</tr>
</tbody>
</table>

**Discussion**

The PDM rats model has been successfully established by verifying the behavioral experimental results, histopathological changes of uterine tissues and levels of endogenous hormones between MCG and NCG. It could be demonstrated that GZFLC exhibited obvious therapeutic effect on PDM through the above verification results.

The comprehensive analysis of the patient's illness and state is the basis of the TCM theory in diagnosis and treatment. Moreover, the interaction between drugs and the drug combination in traditional Chinese prescriptions (TCP) may significantly influence the blood concentration and their PK parameters after administration. Therefore, the PK studies between the normal and pathological model groups are used to elucidate the differences in absorption, distribution, metabolism and excretion of TCP under pathological conditions (Liu et al., 2014). There is a growing consensus that TCP are complex systems, and a single component is insufficient to characterize their PK process in vivo. According to the studies on GA, ALB and AMY, these three compounds exhibited anti-inflammatory and analgesic effects (Kim et al., 2006; Wang et al., 2014; Yang et al., 2013). AMY inhibited the PGE2 synthesis and the nitric oxide (NO) production by suppressing the lipopolysaccharid-stimulated mRNA expressions of cyclooxygenase-2 (COX-2) (Yang et al., 2013). ALB was able to inhibit inducible nitric oxide synthase (iNOS) and COX-2 gene expression induced by lipopolysaccharid (LPS) and the subsequent production of NO and PGE2, protein expression of COX-2 (Wang et al., 2014). GA was a competitive and selective inhibitor of COX-2 (Reddy et al., 2010). PA was the metabolite of ALB and CA exhibited anti-inflammatory effects (Chi et al., 2005; Song et al., 2016). In this study, the PDM rats model was chosen and GA, AMY, ALB, PA and CA were used as the main index bioactive ingredients for investigating PK processes of GZFLC (Zhuo et al., 2015).

In the PK study, there were significant differences in PK parameters between the NTG and PTG rats. PTG rats showed better absorption, distribution and lower elimination of GA, AMY, ALB and PA compared to NTG rats. Pathological status was considered to be the major reason for alteration in PK parameters (Li et al., 2012), which may lead to changes in the function of drug metabolizing enzymes, such as β-glucosidase, cytochrome P450, cytochrome b5, glutathione-transferase, and UDP-glucuronosyl transferase, together with the expression of drug transporters like P-glycoprotein (Gao et al., 2015; Giftson et al., 2011; Gravot et al., 2004; Newmark et al., 1981). In addition, the poor blood circulation in PDM model rats may prolong the retention time of bioactive ingredients of GZFLC in small intestine, which led to the differences of intestinal bioconversion, drug absorption and distribution between the normal and pathological condition (Wen et al., 1995).

The PD results indicated that GZFLC was able to treat PDM rats by increasing the concentrations of PGE2, 6-Keto-PGF1α and reducing the concentrations of TXB2 to normal level. The ratios of PGE2/TXB2 and 6-Keto-PGF1α/TXB2 were conducted for describing PD behaviors more comprehensively and investigating the relationship between plasma concentration and effects more thoroughly and simple. Meanwhile, in order to prevent the rats from being exsanguine, the sampling volume collected every time was approximately 0.3 ml. Since the absorption of CA was too fast and the Tmax value was too small compared with the other four compounds, the PK data for CA could not be fitted with the PD data. Hence, the concentrations of GA, ALB, AMY and PA in rats plasma were selected as the PK markers to map the anti-
dysmenorrhea effect of GZFLC. As showed in Figs. 6 and 7, there were significant delays between the effects and the plasma concentrations of GA, ALB and AMY. The main reason for delays was that the effect site compartment was considered to be different from the central compartment and separated. Moreover, the pharmacological actions induced by active metabolites were another possibility for delays.

The TCP are the main forms of clinical use of TCM. In most cases, TCP are more effective than single herb in terms of safety and efficacy aspects. To clarify the mechanisms of action and combination of TCP and evaluate their efficacy and rationality, the key is to investigate bioactive ingredients in the TCP and their relationship between pharmacokinetics and pharmacodynamics. GZFLC, a classical TCP, is used for the treatment of PDM and other gynecological diseases (Hu et al., 2014; Sun et al., 2016). In order to investigate the relationship between the PK properties of GA, ALB, AMY and PA and the PD effects induced by GZFLC, a simple $E_{\text{max}}$ model with effect site compartment was established in this study, which was one of the most common models for correcting the anti-clockwise hysteresis in the PK-PD relationship (Robinson et al., 2015).

After analyzing the $E_{\text{max}}$ values in PK-PD model results (Table 2), GA, AMY, ALB and PA were found to play the decisive roles in the treatment of PDM, indicating that $\text{Paeonia lactiflora Pall}$, $\text{Moutan Cortex}$ and $\text{Persicae Semen}$ were the main medicinal materials for the treatment of PDM in GZFLC prescription. In addition, the $E_{\text{max}}$ of PA were maximal in both $6\text{-Keto-PGF}_{1\alpha}/\text{TXB}_2$ and $\text{PGE}_2/\text{PGF}_{2\alpha}$ model results, indicating that PA played a more direct and effective role in the treatment of PDM. The $\text{EC}_{50}/\text{C}_{\text{max}}$ values of PA were maximal while the ALB were minimum in both two model results, suggesting that the absorption of ALB was the worst in the effect site compartment while the PA was the best. Since the $k_{01}$ values of these bioactive ingredients were all less than the corresponding $\beta$ values, it could be inferred that the bioactive ingredients would rapidly enter the effect sites from plasma and...
function for a long time. As aforementioned, PA as the metabolite of ALB and bioactive ingredients of *Persicae Semen*, played the most important role in the treatment of PDM. Moreover, as shown in the Figs. 6A and 7A, there was no significant hysteresis between distribution of prunasin in plasma and effect, illustrating that the bioactive ingredients of GZFLC may be metabolized into active metabolites in vivo, and the active metabolites would play more direct and effective roles in the treatment of PDM. Herbal medicines *Persicae Semen* and *Moutan Cortex*, were commonly used to treat blood stagnant syndrome by improving blood circulation and inhibiting blood coagulation in TCM (Ishida et al., 1987; Zhu and Liu, 1992). *Paeonia lactiflora Pall* has been used in relieving various pain in TCM, such as PDM (He and Dai, 2011). Thus, *Persicae Semen* may be the most important medicinal materials for the treatment of PDM in GZFLC prescription by improving blood circulation and inhibiting blood coagulation, while the analgesic effect of *Paeonia lactiflora Pall* was relatively weak due to the poor absorption of ALB in effect site compartment, which may be caused by the interaction between drugs and the drug combination as well as the pathological state of PDM.

Pathophysiology of PDM and the simplified pathway for the biosynthesis of PGs are showed in the Fig. S3. PGs are biosynthesized from ARA through the COX pathway. Phospholipids are hydrolyzed by phospholipase to produce ARA, and then the ARA produces cyclic endoperoxides PGG2 and PGH2 under the action of COX, and finally produces the final products prostacyclin (PGI2), PGF2α, PGE2 and TXA2 under the action of different enzymes. After comparing the E_{max}, EC_{50}, C_{max} and k_{e0} values of GA, AMY, ALB and PA between 6-Keto-PGF1α/TXB2 and PGE2/PGF2α model results, we found that the k_{e0} and EC_{50} values in the 6-Keto-PGF1α/TXB2 model results were greater than those in PGE2/PGF2α model results while the E_{max} values were opposite, which indicated that the amount of these bioactive ingredients transported from the plasma to the effective sites in 6-Keto-PGF1α/TXB2 and PGE2/PGF2α model were less than in PGE2/PGF2α model, resulting in better treatment effect. TXB2 and 6-Keto-PGF1α are stable metabolite from two unstable PGs, TXA2 and PGI2. It could be inferred...
that the active metabolites and prototypes of bioactive ingredients in GZFLC were inclined to regulate the activity of prostacyclin synthetase and thromboxane synthetase to control the production of TXA2 and PGI2, so as to treat PDM by improving blood circulation, preventing platelet aggregation and thrombosis (Dawood and Khan-Dawood, 2007b; Hayes and Rock, 2002). Meanwhile, this inference also proved that *Paeonia Semen, Moutan Cortex* and *Paeonia lactiflora* Pall played the most important role among these three ingredients, while the role of *Paeonia lactiflora* Pall was the weakest.

Conclusions

In this paper, GA, ALB, AMY and PA were selected as the PK markers to map the anti-dysmenorrhea effect of GZFLC after comparing the PK behaviors between the NTG and PTG rats. The hysteresis between plasma concentration-time curves and the corresponding effect-time curves was observed and successfully explained by the introduction of effect site compartment. PK-PD modeling of the anti-dysmenorrhea effect of GZFLC was achieved by a two-compartment PK model with a simple E_{max} PD model. The PK parameters (AUC_{0-}\infty, t_{1/2}, C_{max}, V_{z}/F and MRT) in PTG rats were significantly increased compared with those in NTG rats, which indicated that the pathological status was able to affect the absorption, distribution, metabolism and excretion of GZFLC in vivo. The analysis of PK-PD parameters (E_{max}, EC_{50}/C_{max} and k_{ad}) indicated that the active metabolites and prototypes of bioactive ingredients in GZFLC were inclined to regulate the activity of prostacyclin synthetase and thromboxane synthetase to control the production of TXA2 and PGI2 so as to treat PDM by improving blood circulation, preventing platelet aggregation and thrombosis. As the main effective medicinal materials for the treatment of PDM in GZFLC prescription, and *Paeonia Semen* played the most important role among these three ingredients, while the role of *Paeonia lactiflora* Pall was the weakest. Investigations on the relationship between the effects and the bioactive ingredients are of benefit to explore the mechanisms of action and combination for traditional TCP and facilitate the development of future clinical applications of TCP.

Conflict of interest

The authors declare there is no conflict of interests.

Acknowledgments

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Supplementary materials


References


Table 2
Pharmacokinetic and pharmacodynamic parameters of gallic acid, amygdalin, albi florin and prunasin in PDM treated group (PTG) rats (mean ± SD; n = 6).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Gallic acid</th>
<th>Amygdalin</th>
<th>Albi florin</th>
<th>Prunasin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-Keto-PGF1α</td>
<td>PGE2/PGF2α</td>
<td>6-Keto-PGF1α</td>
<td>PGE2/PGF2α</td>
</tr>
<tr>
<td>k_{ad}</td>
<td>1.89 ± 1.09</td>
<td>1.78 ± 1.85</td>
<td>1.27 ± 1.16</td>
<td>1.58 ± 1.48</td>
</tr>
<tr>
<td>k_{12}</td>
<td>1.59 ± 1.54</td>
<td>1.60 ± 0.68</td>
<td>1.04 ± 1.41</td>
<td>0.79 ± 0.30</td>
</tr>
<tr>
<td>k_{21}</td>
<td>0.11 ± 0.15</td>
<td>0.09 ± 0.17</td>
<td>0.05 ± 0.04</td>
<td>0.06 ± 0.14</td>
</tr>
<tr>
<td>k_{31}</td>
<td>0.44 ± 0.36</td>
<td>0.51 ± 0.45</td>
<td>0.13 ± 0.20</td>
<td>0.39 ± 0.12</td>
</tr>
<tr>
<td>k_{41}</td>
<td>0.86 ± 0.38</td>
<td>0.83 ± 0.44</td>
<td>0.53 ± 0.091</td>
<td>0.44 ± 0.15</td>
</tr>
<tr>
<td>k_{51}</td>
<td>0.27 ± 0.2</td>
<td>0.35 ± 0.33</td>
<td>0.10 ± 0.18</td>
<td>0.35 ± 0.095</td>
</tr>
<tr>
<td>E_{max}</td>
<td>0.035</td>
<td>0.068</td>
<td>0.029</td>
<td>0.055</td>
</tr>
<tr>
<td>l_{max}</td>
<td>1.05</td>
<td>1.94</td>
<td>1.17</td>
<td>1.94</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>302.35</td>
<td>351.35</td>
<td>357.60</td>
<td>393.67</td>
</tr>
<tr>
<td>E_{0}</td>
<td>0.237</td>
<td>1.017</td>
<td>0.237</td>
<td>1.017</td>
</tr>
</tbody>
</table>


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