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Development of an SPE–HPLC–MS method for simultaneous determination and pharmacokinetic study of bioactive constituents of Yu Ping Feng San in rat plasma after oral administration

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Ethnopharmacological relevance: Yu Ping Feng San (YPFS, in Chinese: Jade Windscreen Powder), a well-known traditional Chinese medicine, is commonly used to cure the diseases of respiratory systems and immune systems.

Aim of the study: A selective and sensitive high-performance liquid chromatography coupled with mass spectrometry method (HPLC–MS) was developed and validated for simultaneous quantification of cycloastragenol, formononetin, calycosin, 4'-O-β-glucopyranosyl-5-O-methylvisamminol (GMV) and cimifugin in rat plasma after oral administration of Yu Ping Feng San decoction.

Materials and methods: Plasma samples were extracted via solid-phase extraction (SPE), separated on a Zorbax SB-C18 column, detected by single quadruple mass spectrometry with an electrospray ionization interface, and quantified using selected ion monitoring mode. The current SPE–HPLC–MS assay was validated for linearity, intra-day and inter-day precisions, accuracy, extraction recovery and stability. The method was applied to a comparative pharmacokinetic study after administration of Yu Ping Feng San to rats at different doses (10, 20 and 40 g/kg).

Results: The calibration curves were linear over the ranges 0.50–50 ng/mL and 17.36–1736 ng/mL. Intra- and inter-day precisions (relative standard deviations) were from 0.45% to 10.95%, and accuracy (relative recovery) from 95% to 115%. The extraction recoveries were greater than 88.42% for all analytes. Dose-dependence was shown for some constituents in the drug concentration–time profiles. Among all the active ingredients detected, cimifugin had the highest blood concentration (881–1510 ng/mL), and cycloastragenol had the longest retention time in the rat body (15.06–20.44 h).

Conclusion: This analytical method is a selective, sensitive, precise, accurate, and reliable assay for simultaneous determination of cycloastragenol, calycosin, formononetin, GMV, and cimifugin in rat plasma.

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1. Introduction

Yu Ping Feng San (YPFS, in Chinese: Jade Windscreen Powder) is a well-known formulation used in traditional Chinese medicine (TCM). It consists of a 1:2:2 by weight combination of Saposhnikoviae divaricata Turcz. Schischk, in the Apiaceae, Fang-feng in Chinese), Radix Astragali (Bge. var. mongholicus Fisch. Hsiao or Astragalus membranaceus Fisch. Bge., in the Fabaceae, Huang-qi in Chinese), Rhizoma Atractylodes Macrocephala (Atractylodes macrocephala Koidz, in the Compositae, Bai-zhu in Chinese) (State Pharmacopoeia Committee, 2010). YPFS is useful in the treatment of respiratory diseases such as repeated respiratory tract infections, nosocomial pneumonia, senile chronic bronchitis, and perennial allergic rhinitis (Huang et al., 2004; Li et al., 2007; Wang et al., 2009; Yan et al., 2010). As a safe and effective formulation, it also can relieve idiopathic sweating and augment appetite in end-stage cancer patients (Chiu et al., 2009). It has been used for preventing viral infections including severe acute respiratory syndrome (Lau et al., 2005; Poon et al., 2006), and is reported to have immunoregulatory and beneficial effects in allergic rhinitis, asthma, and dermatitis models (Fang et al., 2005; Makino et al., 2004, 2005; Nakatsuaka et al., 2009). Phytochemical and serum pharmacomedical analyses have found that YPFS contains isoflavones from Astragalosides and chromones from Saposhnikoviae as major constituents (Huang et al., 2008; You, 2007; Zhao et al., 2011; Zheng et al., 2011). In Radix Astragali, astragalosides have been identified as potential bioactive compounds via intestinal absorption and cell extraction studies (Xu et al., 2006; Zhang et al., 2008). Furthermore, numerous pharmacological studies have
shown anti-inflammatory, antiviral, and antioxidant activities for the constituents astragalosides, astragaloside IV, calycosin-7-O-β-D-glucoside, prim-O-glucosylcimifugin and 4′-O-β-glucopyranosyl-5-O-methylvisamminol (Wang et al., 2008; Xue et al., 2000; Yin et al., 2010; Yu et al., 2005; Zhang et al., 2003, 2006). These glycosides (Fig. 1) may explain the effectiveness of YPFS.

In recent years, pharmacokinetic studies of traditional Chinese medicines have received more attention in drug formulation research (Hao et al., 2009; Li et al., 2011; Liu, 2005; Song and Qian, 2008). As reflected by pharmacokinetic profiles, it is accepted that poor bioavailability, low serum concentration, and short retention time indicates that a drug will not be effective. However, according to our preliminary experiments and related literature, traces of astragaloside IV, calycosin-7-O-β-D-glucoside, formononetin-7-O-β-D-glucoside, and prim-O-glucosylcimifugin are in rat plasma (Li et al., 2012; Wen et al., 2008; Ye et al., 2007; Zhang et al., 2007), and cycloastragenol, formononetin, calycosin, and cimifugin are metabolized from their glycosides in vivo (Chen et al., 2011; Li et al., 2012; Yung et al., 2012). These aglycones (Fig. 1) have shown higher biological activities than their glycoside counterparts. For example, cycloastragenol, the common aglycone of astragalosides, has been identified as a telomerase activator and has been used to improve the proliferative response of CD8+ T lymphocytes in HIV-infected patients (Fauce et al., 2008; Ríos, 2010; Yung et al., 2012). Calycosin, one of the metabolites of calycosin-7-O-β-D-glucoside, exhibits more potent antioxidant and antiviral activities than its parent form and other metabolites (Chen et al., 2011; Yu et al., 2005).

Methods for determining the presence of these glycosides in YPFS and in biological samples have utilized high-performance liquid chromatography ultraviolet (HPLC-UV) (Huang et al., 2008; Yang et al., 2007; Ye et al., 2007), HPLC–mass spectrometry (MS) (Dai et al., 2008; Wen et al., 2008), and HPLC-tandem MS (MS/MS) (Zhang et al., 2007). Unfortunately, there are few reports of the
pharmacokinetics of these bioactive metabolites in the human body (Li et al., 2012). For example, to our knowledge, there is no report of an analysis of cycloastragenol in vivo. Therefore it is important to develop a method for quantitative analysis of such bioactive metabolites and their pharmacokinetic properties.

In this study, taking advantage of solid-phase extraction (SPE), we developed and validated a rapid and sensitive method using SPE and liquid chromatography (LC)–MS for simultaneous quantification of cycloastragenol, formononetin, calycosin, 4'-O-β-D-glucopyranosyl-5-O-methylvisamminol (GMV), and cimifugin in rat plasma. We also applied this method to perform a pharmacokinetic study of YPFS decoction administered orally at different concentrations.

2. Materials and methods

2.1. Chemicals and materials

Cycloastragenol, formononetin, calycosin, GMV, and cimifugin were purchased from Zelang Pharmaceutical (Nanjing, China). *Radix Saposhnikoviae*, *Radix Astragali* and *Radix Atractylodis Macrocephalae* were purchased from Tongrentang Chinese Medicine. Since 1669 (Beijing, China) in May, 2011, and were identified by Professor Zhongming Zhou of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences. Voucher specimens (HQ20110522, BZ20110522 and FZ20110522) were deposited in the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences. Methanol was of HPLC grade (Fisher, USA). Trichloroacetic acid was purchased from Sinopharm Chemical Reagent China (Beijing, China). Deionized water was prepared with a Milli-Q system (Millipore, MA, USA). Other chemicals were of analytical grade. SPE cartridges (C18-U, 3 mL/200 mg) were purchased from Dikma Technologies (Beijing, China).

2.2. Apparatus and HPLC–MS conditions

The HPLC–MS system consisted of a surveyor HPLC system (Agilent 1200, USA) and a single quadrupole mass spectrometer (Agilent 6130, USA) equipped with an electrospray ionization source. Data acquisition and analysis were performed with LC/MS Chemstation software (Agilent, USA).

Chromatographic separation was conducted on a Zorbax SB-C18 column (2.1-mm id × 150 mm, 5 μm, Agilent, USA); oven temperature at 35 °C. The mobile phase was methanol/water in a gradient elution, produced by starting at 40:60 v/v, changing to 80:20 v/v over 10 min of elution, then gradually to 100:0 v/v reached at 15 min. This was maintained until 16.5 min, the final ratio of 60:40 v/v was achieved at 17 min. The flow rate was 0.3 mL/min, and the injection volume was 10 μL.

The mass spectrometer was operated in negative mode for formononetin and calycosin and positive mode for cycloastragenol, GMV, and cimifugin. Quantification was obtained using selected ion monitoring mode in multiple channels: cycloastragenol m/z 513.3 [M+Na]+, formononetin m/z 267.0 [M–H]−, calycosin m/z 283.0 [M–H]−, GMV m/z 475.5 [M+Na]+ and cimifugin m/z 307.0 [M+H]+ (Fig. 2). The MS parameters were as follows: flow rate of drying gas (nitrogen) 9.0 L/min, nebulizer pressure 35 psig, drying gas temperature (nitrogen) 350 °C, capillary voltage 3500 V in positive and negative modes, fragmentor voltage 100 eV, and gain setting 1. MS operating conditions were automatically optimized by infusion of the standard solution of each constituent into the electrospray ionization source via a syringe pump.

2.3. Preparation of YPFS decoction

Crude *Radix Saposhnikoviae*, *Radix Astragali*, and *Radix Atractylodis Macrocephalae* were weighed in 6:12:12-g portions and thoroughly soaked in water for 30 min. For the first decoction, 10-fold water (1:10, v/v) was added to the crude drugs and the mixture boiled for 1.5 h, and the decoction was filtered. For a continuation of the decoction preparation, 8-fold water (1:8, v/v) was added to the crude drug residue and it was boiled for 1 h. After filtration, this decoction was combined with the previous one and concentrated to make two different concentrations, 1 and 2 g/mL (crude drugs/decoction). HPLC–MS analysis showed that the percentages of formononetin, calycosin, GMV, and cimifugin in the decoction were 2.0%, 6.2%, 4.6%, and 5.0% (mg/g), respectively.

2.4. Preparation of standard and quality control samples

Stock solutions of cycloastragenol, formononetin, calycosin, GMV, and cimifugin were prepared in methanol. Calibration samples were prepared by spiking blank plasma samples to obtain final concentrations of 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, or 50.0 ng/mL for cycloastragenol, formononetin, calycosin, and GMV, and 16, 32, 64, 160, 320, 640, or 1600 ng/mL of cimifugin. Quality control samples containing 1.5, 7.5, or 30 ng/mL of cycloastragenol, formononetin, calycosin, and GMV, or 50, 250, and 1000 ng/mL for cimifugin were prepared in the same manner. The stock solutions were stored at 4 °C, calibration and quality control samples were prepared when needed.

2.5. Sample preparation

Plasma samples were removed from −80 °C storage and thawed in a water bath under ambient conditions. One milliliter of the homogenized plasma sample was transferred to a 2.0-mL microcentrifuge tube and then 600 μL of 1% trichloroacetic acid was added to precipitate the protein. After vortexing for 2 min and centrifugation at 10,000 rpm for 5 min, the supernatant was then loaded onto the preconditioned SPE cartridge (i.e., preconditioned with 3 mL methanol followed by 3 mL purified water). The cartridge was rinsed twice with 2 mL of water and then centrifuged at 3000 rpm for 5 min to get rid of the residual water in the SPE cartridge. The compounds were eluted with 1.3 mL methanol–water (1:1, v/v). After centrifugation at 10,000 rpm for 5 min, 10 μL of the supernatant was directly injected into the HPLC–MS system for analysis (Fig. 3).

2.6. Method validation

2.6.1. Specificity, linearity and sensitivity

The specificity of the method was assessed by comparing the lowest concentration in the calibration curves with blank rat plasma that had undergone the same pretreatment and analysis.

Calibration curves were generated by plotting the peak area (y-axis) against the plasma concentrations of cycloastragenol, formononetin, calycosin, GMV, and cimifugin (x-axis). The lower limit of quantification and the lower limit of determination were defined as the final concentration producing a signal-to-noise ratio of 10 and 3, respectively. The upper limit of quantification was taken as an in vivo peak concentration of ≥120%.
Fig. 2. MS spectra and the generated ions (A), the selected ion monitoring chromatograms (B) of the constituents in rat plasma after oral administration of Yu Ping Feng San decoction: cycloastragenol (2), formononetin (4), calycosin (6), 4’-O-[l]-glucopyranosyl-5-O-methylvisamminol (9), and cimifugin (8). The most abundant ions were [M+Na]+ at m/z 513.3 generated by cycloastragenol, [M-H]- at m/z 267.0 by formononetin, [M-H]- at m/z 283.0 by calycosin, [M+Na]+ at m/z 475.1 by GMV, and m/z 307.0 [M+H]+ by cimifugin.
2.6.2. Extract recovery, matrix effect, precision, accuracy, and stability

The extraction recoveries were determined using quality control plasma samples, by comparing the peak area obtained from the plasma sample spiked before extraction with those from plasma samples spiked after extraction. The matrix effect was evaluated by comparing the absolute peak area of post-extracted control plasma spiked with a known amount of drug and the areas obtained by direct injection of neat standard samples at equivalent concentrations.

Validations of precision and accuracy were carried out with quality control samples (n = 5) on the same day (intra-day) and on three consecutive validation days (inter-day). The intra-day and inter-day precision results were based on the relative standard deviations, whilst relative recovery (RR) was calculated to evaluate the accuracy.

Evaluation of freeze and thaw stability was carried out by subjecting quality control plasma samples to two freeze (−80 °C) and thaw (in a water bath at room temperature) cycles. Room-temperature stability was evaluated by leaving quality control plasma samples under ambient conditions for 4 h. Post-preparation stability was tested by keeping the same samples under auto-sampler conditions for 0 or 12 h.

2.7. Application of the method and pharmacokinetic study

The studies with rats were all performed in accordance with the protocols required by the Animal Care and Use Committee of the China Academy of Chinese Medical Sciences. Forty-five male Wistar rats (6–7 weeks old), weighing 200 ± 20 g, were purchased from the Institute of Experimental Animals, Academy of Military Medical Sciences (Beijing, China). The rats were randomly and equally divided into three treatment groups (n = 15, each), and fasted overnight (14 to 16 h) prior to oral administration of a low- (10 g/kg body weight, 0.5 g/mL), moderate- (20 g/kg body weight, 1 g/mL) or high- (40 g/kg body weight, 2 g/mL) dose YPFS decoction. Blood samples were collected into heparinized tubes by retro-orbital plexus puncture at 0.5, 1, 2, 4, 8, 12, 24, 36, and 48 h after the oral dose. A kinetic study was carried out; data from at least 5 rats was recorded at each time point. After centrifugation (3000 rpm for 5 min, 4 °C), plasma samples were stored at −80 °C until used for analysis. All pharmacokinetic parameters were processed by non-compartmental analysis using WinNonlin software (Pharsight Corporation, USA).

3. Results and discussion

3.1. Method development

A standard solution of each constituent was directly infused into the electrospray ionization source under the positive ion scan mode and negative ion scan mode. The most abundant ions were [M + Na]⁺ at m/z 513.3 generated by cycloastragenol, [M−H]⁻ at m/z 267.0 by formononetin, [M−H]⁻ at m/z 283.0 by calycosin, [M + Na]⁺ at m/z 475.1 by GMV, and m/z 307.0 [M + H]⁺ by cimifugin (Fig. 2A). Therefore, these ions were selected for quantification using selected ion monitoring mode in multiple channels (Fig. 2B). A gradient elution was employed to reduce retention time and avoid excessive broadening of the peaks. Under the developed chromatographic conditions, the retention times were approximately 16.2 min for cycloastragenol, 10.5 min for formononetin, 7.3 min for calycosin, 6.5 min for GMV, and 4.8 min for cimifugin.

ProElut ™ C18-U SPE cartridges were used for the preparation of plasma samples not only because of their absorption capacity for non-polar compounds but also for polar compounds. Trichloroacetic acid was used to precipitate the protein and the diluted plasma to avoid blocking the SPE cartridge. Polysaccharides, phospholipids, proteins, and interfering compounds can be removed by deionized water.

Table 1

| Constituents      | Regression equation | r²     | Linearity rangea | LLODa | LLOQa |
|-------------------|---------------------|--------|------------------|-------|-------|
| Cycloastragenol   | Y = 3251.1x + 23198 | 0.9998 | 0.54–54.25       | 0.18  | 0.54  |
| Formononetin      | y = 6893.8x + 4418.6 | 1.0000 | 0.51–50.75       | 0.17  | 0.51  |
| Calycosin         | Y = 4278.3x + 2418  | 0.9999 | 0.51–50.75       | 0.17  | 0.51  |
| GMV               | Y = 5606.9x + 3913  | 0.9999 | 0.53–52.50       | 0.18  | 0.53  |
| Cimifugin         | y = 7913.8x + 24820 2 | 0.9940 | 17.36–1736.00     | 0.17  | 0.51  |

Note: a ng/mL; r², square of the correlation coefficient; LLOD, lower limit of determination; LLOQ, lower limit of quantification; GMV, 4′-O-β-glucopyranosyl-5-O-methylvisamminol.
water, reducing the matrix effect of the sample and improving sensitivity to the constituents. Methanol–water was used to reconstitute the dry residue to produce better peaks. In summary, the method of sample preparation achieved satisfactory results simultaneously for the five constituents.

3.2. Method validation

Based on the results of the trial test, the respective lower limits of determination for cycloastragenol, formononetin, calycosin, GMV, and cimifugin were set at 0.18, 0.17, 0.17, 0.18, and 0.17 ng/mL; the lower limits of quantification at 0.54, 0.51, 0.51, 0.53, and 0.51 ng/mL; and the upper limits of quantification at 54.25, 50.75, 50.75, 52.50, and 1736.00 ng/mL (Table 1). All standard curves exhibited good linearity in the selected ranges.

The square of the correlation coefficient (r²) for cycloastragenol, formononetin, calycosin, GMV, and cimifugin were 0.9998, 1.0000, 0.9999, 0.9999, and 0.9940, respectively.

The extraction recoveries determined for all analytes at three concentrations were ≥88.42%, with relative standard deviation values ≤10.38%. The matrix effects ranged from 88.08% to 113.17%, with relative standard deviation values ≤9.20% (Table 2).

The precision relative standard deviation values for intra-day and inter-day ranged from 3.19% to 10.95% in the quality control samples; the accuracy (i.e., relative recovery, RR) ranged from 95% to 115% (Table 3).

The stability results showed that all analytes were stable in plasma samples after 4 h at room temperature (RR: 85.41–110.75%), 12 h under auto-sampler conditions after preparation (RR: 88.41–102.02%), and two freeze–thaw cycles (RR: 92.73–104.96%), except for calycosin at the lowest level (Table 4). Thus, subjecting samples to repeated freeze–thaw cycles should be avoided.

3.3. Results of pharmacokinetic study

We validated the method as described herein and successfully used it to quantify five constituents of YPFS in plasma samples after oral administration of YPFS decoction at three doses. YPFS was well-tolerated by all subjects with no apparent dose or time dependent toxic effects. The pharmacokinetic parameters terminal half-life (H_{\text{t,0.5}}), maximum concentration in plasma (C_{\text{max}}), and the area under the plasma concentration–time curve (AUC) from dosing time extrapolated to infinity (AUC_{\text{0-\infty}}) showed a trend of dose-dependence towards the detected constituents cycloastragenol, calycosin, GMV and cimifugin in YPFS, based on the doses administered orally (10, 20 and 40 g/kg; Fig. 4, Table 5). Formononetin, calycosin, and GMV were rapidly absorbed, achieving C_{\text{max}} in less than 30 min. Cycloastragenol was detected after 0.5 h in plasma and with time to C_{\text{max}} (i.e., T_{\text{max}}) at around 12 h, a long mean residence time (MRT) of 17 h. We speculated that the astragalosides were hydrolyzed to cycloastragenol in the intestine or stomach, and then cycloastragenol was absorbed into the circulation. An unknown peak (17.2 min) was found in the same selected ion monitoring (513) channel one minute after the cycloastragenol position. It displayed similar pharmacokinetic behaviors as cycloastragenol. This unknown peak will be identified in a further study. Cimifugin showed multiple absorption peaks, and among all the detected constituents the concentration in plasma was particularly high (Fig. 4). This might be because the YPFS contained free cimifugin as well as its precursor.

Since these constituents, especially cycloastragenol and cimifugin, showed some dose-dependence in the pharmacokinetic profiles, these results provide helpful understanding of the therapeudic process of YPFS; however, it showed no obvious dose-dependence...
for formononetin over the approved dosage range, administration of a larger range of doses warrants further study. Previously it was shown that astragalosides from *Radix Astragali* have low permeability *in vitro* and poor bioavailability *in vivo* (Gu et al., 2004; Wen et al., 2008). However, its aglycone cycloastragenol can be absorbed through intestinal epithelium efficiently (Zhu et al., 2010). This is similar to prim-O-glucosylcimifugin, 4'-O-β-glucopyranosyl-5-O-methylvisamminol, and their aglycone cimifugin (Li et al., 2010; Xue et al., 2000; Zhao et al., 2011). It has been reported that changes in telomere lengths are relevant to immune function (Weng, 2012), and

### Table 4
Stability of the constituents in YPFS in rat plasma and prepared samples (*n* = 3).

|                | Spiked (ng/mL) | Stability in rat plasma samples | Stability in prepared samples |
|----------------|----------------|---------------------------------|-------------------------------|
|                | Freeze–thaw (ng/mL) | RR (%) | 4 h (ng/mL) | RR (%) | 0 h (ng/mL) | 12 h (ng/mL) | RR (%) |
| **Cycloastragenol** | 1.63 | 1.55 ± 0.02 | 95.24 | 1.39 ± 0.06 | 85.41 | 1.47 | 1.36 | 92.52 |
|                | 8.14 | 8.40 ± 0.10 | 103.23 | 9.28 ± 0.18 | 114.04 | 9.61 | 9.10 | 94.69 |
|                | 32.55 | 33.13 ± 0.28 | 101.78 | 32.96 ± 0.44 | 101.26 | 33.13 | 33.83 | 102.02 |
| **Formononetin** | 1.25 | 1.47 ± 0.01 | 96.55 | 1.50 ± 0.03 | 104.43 | 1.64 | 1.45 | 88.41 |
|                | 7.61 | 8.09 ± 0.09 | 106.27 | 8.02 ± 0.09 | 105.35 | 9.17 | 8.86 | 96.62 |
|                | 30.45 | 31.96 ± 0.12 | 104.96 | 31.62 ± 0.25 | 103.84 | 31.77 | 31.04 | 97.70 |
| **Calycosin** | 1.52 | 0.99 ± 0.03 | 65.02 | 1.66 ± 0.04 | 109.03 | 1.71 | 1.58 | 92.40 |
|                | 7.61 | 7.72 ± 0.05 | 101.15 | 8.13 ± 0.17 | 106.80 | 9.07 | 8.69 | 95.81 |
|                | 30.45 | 31 ± 0.18 | 101.81 | 29.79 ± 0.66 | 97.83 | 32.92 | 31.88 | 96.84 |
| **GMV** | 1.58 | 1.47 ± 0.12 | 93.33 | 1.43 ± 0.06 | 90.79 | 1.57 | 1.47 | 93.63 |
|                | 7.88 | 8.19 ± 0.20 | 104.00 | 7.80 ± 0.09 | 99.05 | 9.02 | 9.00 | 99.78 |
|                | 31.5 | 32.85 ± 0.03 | 104.29 | 29.20 ± 0.35 | 92.70 | 33.65 | 33.44 | 99.38 |
|                | 260.4 | 217.15 ± 1.76 | 105.36 | 208.1 ± 1.49 | 110.75 | 316.89 | 302.55 | 95.45 |
| **Cimifugin** | 52.08 | 52.70 ± 0.91 | 101.19 | 50.96 ± 0.52 | 97.89 | 52.11 | 50.29 | 96.51 |
|                | 1041.6 | 965.91 ± 11.69 | 92.73 | 995.3 ± 6.35 | 95.55 | 1001.5 | 992.3 | 99.08 |

RR: relative recovery; GMV, 4'-O-β-glucopyranosyl-5-O-methylvisamminol.

**Fig. 4.** Mean concentration–time profiles of cycloastragenol, formononetin, calycosin, 4'-O-β-glucopyranosyl-5-O-methylvisamminol (GMV), and cimifugin in rat plasma after oral administration of *Yu Ping Feng San* decoction at doses 10 g/kg (Low), 20 g/kg (Moderate), and 40 g/kg (High) in rats (mean ± SD, *n* = 5).
cycoastragenol upregulates telomerase activity (Fauce et al., 2008; Rios, 2010; Yang et al., 2012). Furthermore, cimifugin inhibits nitric oxide production and contributes to the analgesic efficacy of *Cynanchum* and *Cicuta* (Okuyama et al., 2001; Wang et al., 1999). Taken together, cycloastragenol and cimifugin have demonstrated good pharmacokinetic behavior in vivo. Therefore, cycloastragenol and cimifugin may be related to the effect of *YPFS* in diseases of the respiratory and immune systems. Moreover, these two metabolites may be valuable as immunoregulatory and anti-inflammatory agents. However, further study is required to confirm the association between cycloastragenol, cimifugin, and *YPFS* and pharmacological effect, as such bioactive metabolites could be potential drugs.

4. Conclusions

In the present work, a SPE–HPLC–MS method for simultaneous quantification of plasma concentrations of five constituents of *YPFS* was developed and validated. We demonstrated that the method is selective, sensitive, precise, accurate, and reliable for simultaneous determination of cycloastragenol, calycosin, formononetin, GMV, and cimifugin in rat plasma samples. In addition, a pharmacokinetic study of *YPFS* was applied with different doses.

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Table 5

| Constituents | Dose | Hmax (h) | Tmax (h) | Cmax (ng/mL) | AUC0-τ (h ng/mL) | AUC0-C0 (h ng/mL) | MRT0-τ (h) |
|--------------|------|----------|---------|--------------|-----------------|------------------|-------------|
| Cycloastragenol | Low | 9.43 8 | 7.65 | 126.85 | 2266.91 | 17.87 |
| Moderate | 6.44 12 | 15.08 | 244.36 | 3681.02 | 15.06 |
| High | 10.69 12 | 19.29 | 440.22 | 8998.31 | 20.44 |
| Formononetin | Low | 8.2 0.5 | 1.52 | 14.9 | 181.86 | 12.21 |
| Moderate | 4.91 0.5 | 5.01 | 29.19 | 124.31 | 7.54 |
| High | 5.03 0.5 | 3.54 | 16.65 | 8.07 |
| Calycosin | Low | 5.17 0.5 | 6.61 | 44.6 | 341.08 | 7.65 |
| Moderate | 7.55 0.5 | 17.79 | 101.15 | 1004.15 | 9.93 |
| High | 4.91 0.5 | 5.01 | 29.19 | 124.31 | 7.54 |
| GMV | Low | 1.32 0.5 | 1.87 | 3.22 | 6.26 | 1.94 |
| Moderate | 1.99 0.5 | 4.88 | 12.35 | 35.11 | 2.84 |
| High | 2.9 0.5 | 22.84 | 18.74 | 68.01 | 3.63 |
| Cimifugin | Low | 1.98 0.5 | 881.33 | 5615.82 | 26614.43 | 4.74 |
| Moderate | 2.88 2 | 1320.75 | 10604.19 | 54774.79 | 5.17 |
| High | 5.95 2 | 1509.62 | 14911.16 | 99798.06 | 6.69 |
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