



Original Article

A reliable and rapid pharmacokinetic study of pueraria isoflavones using pueraria reference extractive substance in beagle plasma: Application to study of Yufeng Ningxin Tablets

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ABSTRACT

Objective: In order to evaluate the reliability and feasibility of pueraria reference extractive substance (RES) used in biological sample, the pharmacokinetics of 3'-hydroxy puerarin (3'-HP), puerarin, 3'-methoxy puerarin (3'-MP), and daidzein-8-C-*apiosyl*-(1-6)-glucoside (DAG) in beagle plasma following oral administration of Yufeng Ningxin Tablet were quantitated.

Methods: A reliable and sensitive high-performance liquid chromatography-tandem triple quadrupole mass spectrometry (HPLC-QQQ-MS/MS) method developed with chromatographic separation was operated on a Merck C₁₈ column, and acetonitrile-5 mmol/L ammonium was used as mobile phase in gradient elution. The plasma samples were deproteinized by acetone, detected by triple quadrupole mass spectrometry with an electrospray ionization interface, and quantified using selected ion monitoring mode. The pharmacokinetic parameters were calculated by Winnonlin 4.1.

Results: The calibration curves of the reference extractive substance and standard substance methods were linear over the ranges 0.0417–11.3309 µg/mL and 0.0394–10.0000 µg/mL. The intra-day and inter-day precision of the two methods at three concentrations were less than 13.63%, and the average recoveries of 3'-HP, puerarin, 3'-MP, and DAG were more than 70.67%. The RSD of the mean plasma concentrations of the analytes calculated by the two methods was less than 5%, and $\cos(\theta) = 1.000$. Among the analytes, puerarin showed the highest blood concentration [(940 ± 185) ng/mL] and the longest retention time [(5 ± 1) h] in the dog's bodies.

Conclusion: Pueraria reference extractive substance can be seen as an alternative to the standard substance to overcome the scarcity of standard substance for the analysis of biological samples.

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1. Introduction

One of the main challenges of compounds identification, inspection, and determination analysis is the scarcity of standard substance (SS). At the same time, the lacking of SS is often a bottleneck in traditional Chinese medicine (TCM) metabolic analysis. Thus the strategy of simultaneous determination of multiple components by reference extractive substance (RES) quantitative method as an alternative to SS quantitative method has been proposed (Jing et al., 2013; Lu et al., 2013; Yuan, Gao, Wang, & Zhang,

2012), which may be comprehensive if suitable method has been established and validated, even if could not be available for SS.

Yufeng Ningxin Tablet (YNT) is a formulation of total isoflavones obtained from roots of *Pueraria lobata* (Willd.) Ohwi, which is recorded in the Chinese Pharmacopoeia (Chinese Pharmacopoeia, 2015). The YNT mainly consist of puerarin and other active isoflavones, such as 3'-HP, 3'-MP, DAG, and daidzin, which contribute to a range of pharmacological effects, e.g. cardiovascular, neurological, hyperglycemic (Hsu et al., 2003; Zhu et al., 2004), anti-oxidative (Guerra et al., 2000), anti-thrombotic, anti-allergic (Choo, Park, Yoon, & Kim, 2002), and potential anti-diabetic effects (Vedavanam, Sriyanta, O'Reilly, Raman, & Wiseman, 1999). YNT is being used increasingly in clinics as a result of the globally grow-

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ing incidence of cardiovascular and cerebrovascular diseases. Till now, several studies about pueraria isoflavones using SS quantitative method have been reported (Xiao et al., 2016; Zhao, Zhao, Liu, Han, & Yu, 2012). In our study, we have attempted to establish a validated RES quantitative method applied to pueraria isoflavones analysis.

RES is prepared using a specific extraction process and contains a variety of main active ingredients or index components, which can be used for the identification or content determination of TCM materials, including slices, extracts, and Chinese patent medicine (Chen, Jin, Sun, Pang, & Ma, 2016; Chinese Pharmacopoeia, 2010). Using RES instead of SS might provide a way of resolving the problem of lack of the SS.

Although several pharmacokinetic studies have focused on puerarin, daidzin or daidzein individually (Li, Deng, Wang, Qiao, & Li, 2013; Liu et al., 2013; Wang et al., 2012; Yan, Xing, Ding, Tao, & Du, 2004; Yu, Shia, Tsai, & Hou, 2012), there are no reports on pueraria RES method which applied to the determination of active ingredients in biological samples or the study of pharmacokinetics. In this report, we described a quantitative method using the RES that can be used to simultaneously quantify 3'-HP, puerarin, 3'-MP and DAG in beagle dog plasma after oral administration of YNT. Comparing with the previous reports, we successfully apply the RES to quantity several compounds in biological samples, which provides a new strategy for the analysis of biological samples.

2. Materials and methods

2.1. Chemicals and materials

Liquiritin (IS, Batch No. 111610-201607, purity 93.1%) and puerarin (Batch No. 110752-201615, purity 95.4%) were purchased from the National Institute for Food and Drug Control of China (NIFDC, Beijing, China). The chemical compounds 3'-HP, 3'-MP and DAG were prepared in our laboratory at the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences (all purity > 96%, determined by high-performance liquid chromatography, HPLC). The RES, consisting of 47.85% puerarin, 10.76% 3'-HP, 9.79% 3'-MP, and 8.80% DAG, was prepared in our laboratory from the dried roots of *Pueraria lobata* (Willd.) Ohwi.

Ammonium acetate (HPLC-grade, Batch No. 20140301) and ammonium formate (Analysis-grade, Batch No. 20140524) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (USA). Ethanol was obtained from Beijing Chemical Reagent Factory (Beijing, China). Water from a Milli-Q water system (Millipore Corp., Bedford, USA) was used to prepare the mobile phase. YNT was produced by the Beijing Tongrentang Technology Development Pharmaceutical Factory (Production Batch No. 12120227).

2.2. Preparation of pueraria RES

The roots of *P. lobata* (1 kg) were extracted in eight times the quantity of 60% ethanol while refluxing for 2 h. The crude extract solution was evaporated under reduced pressure to recover the ethanol and then diluted with water to 3 L. The diluted solution was separated in a column of macroporous resin AB-8 (3 kg, Tianjin Nankai University Resin, Tianjin, China), using different concentrations of ethanol as the eluting solvent. After stepwise elution with 5% ethanol (volume percent, 3 times the bed volume, 3 BV) to remove non-target components, the sample was collected in 60% ethanol (volume percent, 3 BV). The collected solution was evaporated under reduced pressure to reduce the ethanol, and then redissolved in 500 mL water. It was then purified using macroporous resin and chromatographed on a polyamide column (30–60 mesh,

Shanghai Bang Jing Industrial, Shanghai, China). Distilled water (volume percent, 2 BV) was used to elute non-target components and the sample was collected in 30% ethanol (volume percent, 3 BV). The supernatant was purified using polyamide and mixed with 50 g of silica gel (160–200 mesh, Qingdao Haiyang Chemical, Qingdao, China). The mixed powder was evaporated to dryness, pulverized, and chromatographed on a silica gel column with a chloroform-methanol system as the eluting solvent. Following stepwise elution with chloroform-methanol (3:1, volume percent, 2 BV) to remove non-target components, the total RES solution was obtained in chloroform-methanol (2:1, volume percent, 3 BV). The extractive solution was then evaporated under reduced pressure to recover the solvent and obtain the pueraria RES, which was determined by HPLC and standardized in our laboratory, consisting of 47.85% puerarin, 10.76% 3'-HP, 9.79% 3'-MP, and 8.80% DAG.

2.3. Apparatus and HPLC-QQQ-MS/MS conditions

The HPLC-QQQ-MS/MS system consisted of an HPLC system (Agilent 1260, USA) and a triple quadrupole mass spectrometer (Agilent 6460, USA). Data acquisition and analysis were performed using Agilent Chemstation software (Agilent, USA).

Chromatographic separation was conducted on a Merck C₁₈ (2.0 mm × 50 mm, 3 μm) with a pre-column Agilent Zorbax SB-C₁₈ column (4.6 mm × 12.5 mm, 5 μm, Agilent, USA). The column temperature was 30 °C, and the mobile phase consisted of system A (contained 5 mmol/L ammonium formate water) and system B (acetonitrile). A gradient elution was used, which increased from 13% B to 53% B over 10 min and reached 100% B within 5 min, which was maintained for 1.5 min. The flow rate was 0.2 mL/min and the injection volume was 2 μL.

The mass spectrometric detection was performed using an electrospray ionization (ESI) source in the negative ionization mode. The optimized MS/MS parameters were as follows: drying gas (nitrogen) 5.0 L/min, capillary voltage 3.5 kV, nebulizer pressure 45 psi, drying gas temperature 300 °C. Quantification was performed using the Multiple Reaction Monitoring (MRM) detection mode, and the fragment voltage and collision energy were optimized for each target compound.

2.4. Preparation of standard and quality control samples

Methanol stock solutions of RES and SS containing the four pueraria isoflavones (3'-HP, puerarin, 3'-MP, and DAG) and the IS stock solutions were prepared and serially diluted to provide working standard solutions of appropriate concentrations to establish individual calibration curves. At least six concentrations of each analyte were analyzed in duplicate, and the calibration curves were constructed by plotting the peak areas versus the concentrations of each analyte. The quantitation of each analyte was performed based on its individual calibration curve. All solutions were kept at 4 °C.

The calibration standard and quality control (QC) of RES plasma samples were prepared by spiking the RES and IS solutions in blank plasma. The RES concentrations in the plasma were in the range 0.0510–10.1919 μg/mL for 3'-HP, 0.0567–11.3309 μg/mL for puerarin, 0.0464–9.2731 μg/mL for 3'-MP, and 0.0417–8.3354 μg/mL for DAG, respectively. The IS solution in plasma had a concentration of 1.54 μg/mL. QC samples of RES at low, middle and high concentrations (0.20, 2.55, 10.19 μg/mL for 3'-HP; 0.23, 2.27, 11.33 μg/mL for puerarin; 0.19, 2.23, 9.27 μg/mL for 3'-MP; and 0.17, 2.08, 8.34 μg/mL for DAG) were also prepared.

The calibration standard and QC of SS plasma samples were prepared using the same procedures. The low, middle and high concentrations of the QC samples of SS were 0.20, 2.50, 10.00 μg/mL for 3'-HP; 0.19, 2.37, 9.48 μg/mL for puerarin; 0.17,

Table 1
MRM parameters for detection of analytes.

Compounds	RT /min	Precursor ion (m/z)	Production (m/z)	Collision energy /eV	Frag / V
3'-HP	2.55	431	311	20	200
Puerarin	3.98	415	295	20	190
3'-MP	4.34	445	325	20	200
DAG	4.48	547	295	30	225
Liquiritin	8.71	417	135	30	170

2.10, 8.40 µg/mL for 3'-MP; And 0.16, 1.97, 7.88 µg/mL for DAG. Calibration and QC samples were prepared when needed.

2.5. Preparation of plasma samples

Plasma samples were removed from -80°C storage and thawed in a water bath under ambient conditions. The plasma samples (200 µL), 100 µL of IS solution and 100 µL of 5 mmol/L ammonium acetate solution were placed in 5 mL Eppendorf tubes (EP tubes) and mixed, after 600 µL of acetone being added to precipitate the proteins. The mixtures were vortexed for 5 min and centrifuged at 12 000 r/min for 5 min. The supernatant (0.7 mL) was transferred into another EP tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 200 µL methanol-water (50:50, volume percent), vortexed for 5 min, and centrifuged at $12\ 000 \times g$ for 5 min. The supernatant was transferred into another EP tube and filtered through a 0.22 µm Millipore filter before HPLC-QQQ-MS/MS analysis.

2.6. Method validation

Both RES and the SS methods were validated using appropriate indices, including specificity, selectivity, calibration curves, sensitivity, precision, accuracy, matrix effect, recovery rate, and stability, in accordance with the US Food and Drug Administration (FDA) guidance regarding bio-analytical method validation.

2.6.1. Specificity, linearity and low limit of quantitation (LLOQ)

The specificity of the methods was evaluated by comparing the chromatograms of blank plasma from five individual beagles, plasma samples spiked with the analytes and IS, and plasma samples were obtained after administration. Blank plasma was analyzed for endogenous interference.

Calibration curves were constructed from the peak area ratios of analytes to the IS using weighted least-squares linear regression with a weighting factor of $1/x^2$. The lower limit of quantitation (LLOQ) of this method was defined as the lowest concentration on the calibration curve with an acceptable accuracy (relative error, Er) within $\pm 20\%$ and a precision (relative standard deviation, RSD) below 20%.

2.6.2. Precision and accuracy

The intra- and inter-batch precision were evaluated by quantifying three concentrations (high, medium, and low) of QC samples ($n = 5$) on the same day (intra-day) and on three consecutive validation days ($n = 15$, inter-day) by using RSD, respectively. Accuracy was evaluated using the relative error.

2.6.3. Extract recovery rate and matrix effect

The extraction recovery rates and matrix effects of the analytes were determined for five replicates at three different concentrations (low, medium and high levels). The extraction recovery rates of this method were calculated by comparing the peak areas of the extracted QC samples with those obtained from pure reference standards spiked in post-extracted blank dog plasma at the same concentration. The matrix effect was evaluated by comparing the

peak area of post-extracted control plasma spiked with a known quantity of the analytes with the areas obtained by direct injection of pure standard solutions at equivalent concentrations.

2.6.4. Stability

The plasma samples were freshly prepared and stored at -80°C for 30 d to evaluate long-term stability. The freeze-thaw stability of the samples after subjecting them to three freeze-thaw cycles (-80°C to room temperature). The post-preparation stability was tested by determining the extracted stability of plasma samples stored at room temperature for 24 h.

2.7. Pharmacokinetic study

The validated methods were used to determine the plasma concentrations of 3'-HP, puerarin, 3'-MP and DAG in male beagles after a single oral administration of YNT. The YNT dose was 130.9 mg/kg (equivalent to 1.50 mg/kg of 3'-HP, 8.78 mg/kg of puerarin, 1.92 mg/kg of 3'-MP, and 2.17 mg/kg of DAG).

The studies with beagles were all performed in accordance with the protocols required by the Animal Care and Use Committee of the China Academy of Chinese Medical Sciences. Five healthy male beagles, weighing about 12.0 kg, were provided by Institute of Experimental Animals, Shahe Tongli Test Farm, Beijing, China. The dogs were housed under standard conditions and had ad libitum access to water and a standard laboratory diet. They fasted overnight (12 h) prior to the administration of the YNT and resumed feeding 4 h post-dose. Blood samples (approximately 0.3 mL) were taken from the foreleg vein and placed in heparinized test tubes before dose administration and at 0, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240, 480, 720, and 1440 min post-dose. Plasma was separated by centrifugation at 12 000 r/min for 10 min and stored at -80°C until analysis. All pharmacokinetic parameters were processed using non-compartmental analysis with WinNonlin software (Pharsight Corporation, USA). The pharmacokinetic parameters were compared using SPSS 16.0 (Statistical Package for the Social Sciences).

3. Results

3.1. Optimization of HPLC-QQQ-MS/MS conditions

Due to the complexity of TCM preparations, many analogs may be co-eluted during analyses (Yan et al., 2014). In order to develop a sensitive and accurate analytical method, the MRM mode was developed. We preferred the negative mode based on the investigation of both negative and positive ionization modes, because it provided a clearer spectrum. Fragmentation was initiated using nitrogen as the collision gas, and the collision energy was optimized for a stable response and to maximize the intensity of the fragmentation of the analytes and IS. The optimized MS/MS transitions and energy parameters of the analytes and IS were shown in Table 1.

The chromatographic conditions were optimized to improve peak shape, increase signal response, and shorten run time. First, the mobile phase systems of acetonitrile-water and methanol-water in different proportions were investigated. We found that

the acetonitrile–water system produced better peak shapes than the methanol–water system. In addition, it was already well known that additives in the mobile phase could significantly improve ionization efficiency and enhance the response of analytes. Different quantities of ammonium formate (5 mmol/L and 10 mmol/L) and acetic acid (0.1%) were tested to identify the optimal mobile phase. Adding 5 mmol/L of ammonium formate to the water resulted in the greatest response from all of the analytes. Finally, a gradient elution method to achieve good chromatograph spectra was used.

3.2. Method validation

3.2.1. Selectivity, linearity, and LLOQ of RES and SS methods

There was no significant endogenous interference with respect to the retention time of the analytes or IS (Fig. 1), which suggests that the MRM mode is a suitable method for biological analysis.

Fig. 2 showed the typical chromatograms of blank plasma, blank plasma spiked with the RES and IS, and blank plasma spiked with the SS and IS, and plasma sample after YNT administration. All standard curves exhibited good linearity in the selected ranges. The

square of the correlation coefficient (r^2) for every calibration curve was higher than 0.9962. Regression equations for the calibration curves and LLOQs for the five analytes were listed in Table 2.

3.2.2. Precision and accuracy of RES and SS methods

The intra- and inter-day precision of the RES method was in the ranges of 1.20%–13.63% and 1.31%–9.20%, respectively (Table 3). The accuracy was between –13.83% and +13.13% for each QC level of the analytes. All of these were within the acceptable range.

The intra- and inter-day precision of the SS method was 1.09%–10.41% and 0.07%–8.07%, respectively, and the accuracy was between –13.72% and +9.85% for each QC level of the analytes (Table 3), all of which were within the acceptable range. The precision and accuracy of the two methods indicated the measurement was accurate and reproducible.

3.2.3. Recovery rate and matrix effects of RES and SS methods

The extraction recovery rates of 3'-HP, puerarin, 3'-MP, DAG, and IS were stable (Table 4). The matrix effect was between $(89.35 \pm 6.45)\%$ and $(104.34 \pm 7.23)\%$, which is within the acceptable range (Table 4). Thus, the extraction recovery rates were con-

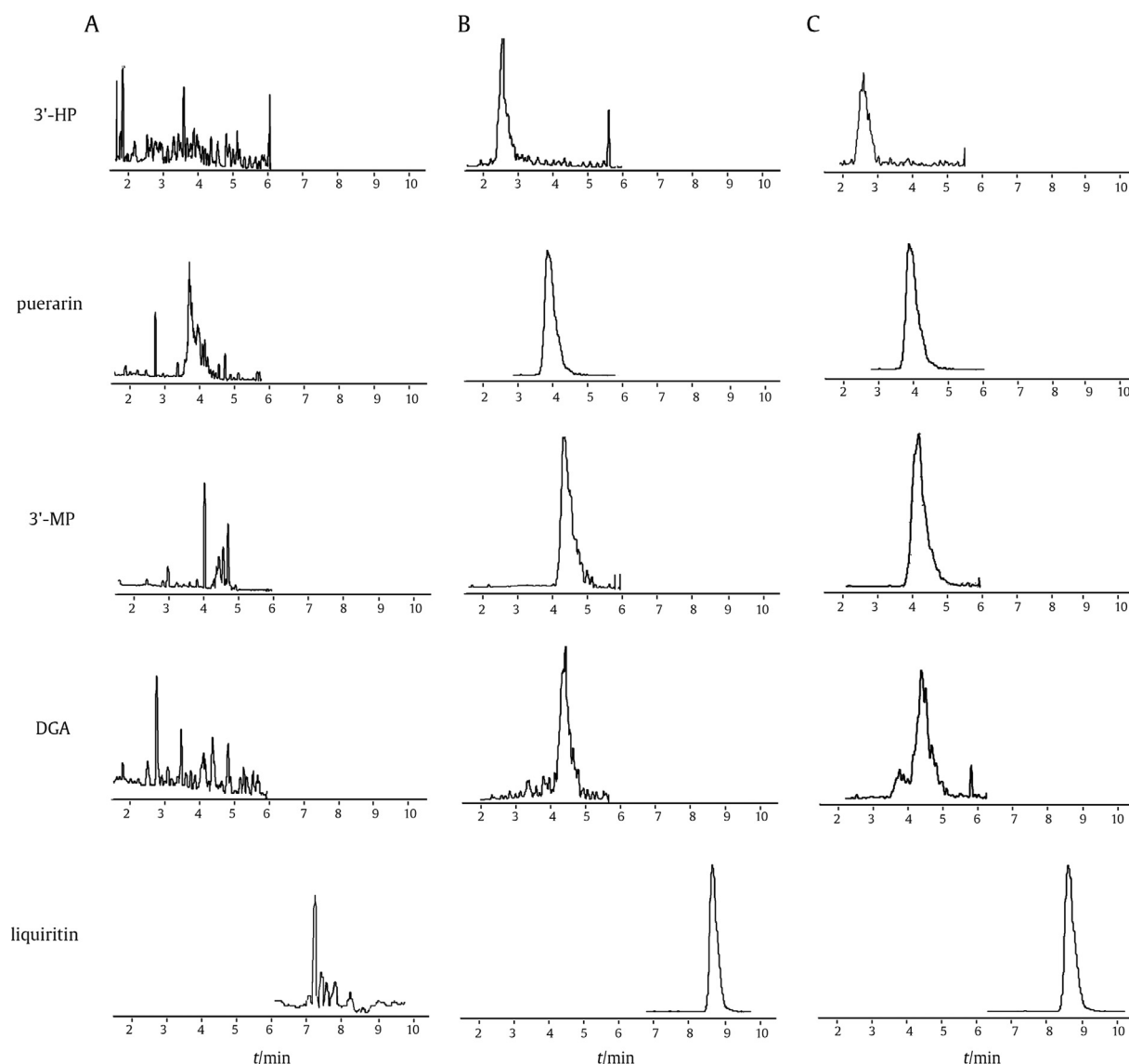


Fig. 1. Representative extract ion MRM chromatograms of pueraria isoflavones and liquiritin. Blank plasma (A), blank plasma spiked with four analytes and IS (B), and plasma sample collected 2 h after a single oral administration of YNT (C).

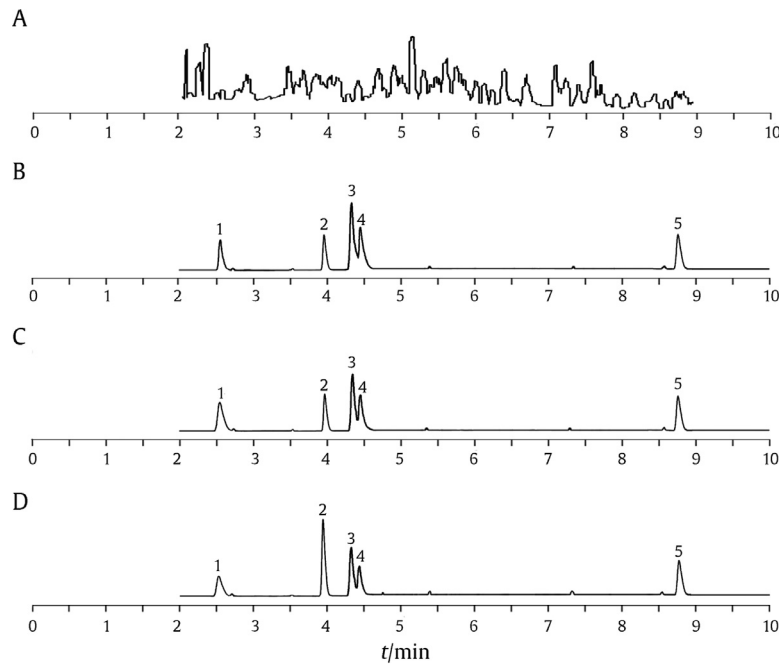


Fig. 2. HPLC of blank plasma (A), blank plasma spiked with RES and IS (B), and blank plasma spiked with SS and IS (C), and plasma sample after YNT administration (D).

Table 2

Calibration curves, linear ranges, and lower limits of quantitation (LLOQ) of four analytes using RES and SS methods ($n = 6$).

	Compounds	Regression equation	r^2	Linear range $/(\mu\text{g}\cdot\text{mL}^{-1})$	LLOQ $/(\mu\text{g}\cdot\text{mL}^{-1})$
RES method	3'-HP	$Y = 0.2027x + 0.0014$	0.9994	0.0510–10.1919	0.0510
	Puerarin	$Y = 0.5982x + 0.1917$	0.9969	0.0567–11.3309	0.0567
	3'-MP	$Y = 0.3450x + 0.0551$	0.9971	0.0464–9.2731	0.0464
	DAG	$Y = 0.2513x + 0.0162$	0.9963	0.0417–8.3354	0.0417
SS method	3'-HP	$Y = 0.2156x + 0.0012$	0.9996	0.0500–10.0000	0.0500
	Puerarin	$Y = 0.6172x + 0.1481$	0.9963	0.0474–9.4800	0.0474
	3'-MP	$Y = 0.3579x + 0.0733$	0.9962	0.0420–8.4000	0.0420
	DAG	$Y = 0.2622x + 0.016$	0.9981	0.0394–7.8800	0.0394

sistent, and matrix effects can be ignored. There were no significant differences between the recovery rate and matrix effects of the RES and SS methods ($P > 0.05$).

3.2.4. Stability of RES and SS

During sample storage and processing, both the RES and SS analytes were stable for one month of storage at $-20\text{ }^{\circ}\text{C}$, for 24 h at room temperature, and through three freeze-thaw cycles. The accuracy and precision of the RES analytes were from -14.21% to $+13.73\%$ and 0.21% – 13.92% , respectively, for one month of storage at $-80\text{ }^{\circ}\text{C}$; From -1.37% to $+14.14\%$ and 0.08% – 11.95% , respectively, for 24 h at room temperature; from -13.42% to $+13.84\%$ and 0.48% – 12.99% , respectively, for three freeze-thaw cycles. The accuracy of the SS analytes for one month of storage at $-80\text{ }^{\circ}\text{C}$, for 24 h at room temperature and through three freeze-thaw cycles, respectively, was from -12.49% to $+14.50\%$, from -13.23% to $+14.52\%$, and from -14.94% to $+13.56\%$, and the precision was 0.23% – 13.42% , 1.07% – 12.90% , and 0.48% – 12.19% , respectively. The accuracy and precision of both the RES and SS analytes indicated the acceptable stability under the above condition.

3.3. Analysis of plasma samples and comparison of RES and SS methods

We compared the consistency of the measurements using the plasma concentrations obtained from the RES and SS methods under the same conditions. All of the isoflavones were detected in

the dog plasma. The mean plasma concentrations of 3'-HP, puerarin, 3'-MP, and DAG were calculated using the two methods.

Relative standard deviation (RSD) and $\cos(\vartheta)$, or the cosine similarity between two vectors, were used to evaluate the consistency of the results obtained from the two methods. The calculation of $\cos(\vartheta)$ was as follows:

$$\cos(\vartheta)(X, Y) = \frac{\sum_{i=1}^n X_i Y_i}{\sqrt{\sum_{i=1}^n (X_i)^2} \sqrt{\sum_{i=1}^n (Y_i)^2}} \quad (1)$$

X and Y represent the plasma concentrations from the RES and SS methods, respectively, and n is the number of datasets.

The RSD of the mean plasma concentrations of 3'-HP, puerarin, 3'-MP, and DAG calculated by the two methods was less than 5%, and $\cos(\vartheta) = 1.000$.

3.4. Pharmacokinetic study

The RES method was successfully used to determine the plasma concentrations of four active constituents of YNT. The major pharmacokinetic parameters of the pueraria isoflavones were calculated using a non-compartment model (Table 5).

Mean (\pm SD) plasma concentration-time curves of isoflavones were illustrated in Fig. 3.

4. Discussion

The pharmacokinetic parameters revealed that the rank order of the flavonoids in terms of maximum concentration (C_{\max}) and

Table 3
Intra- and inter-day precision and accuracy of RES method and SS method for determination of four analytes from assay samples (mean \pm SD).

Compound Spiked $(\mu\text{g}\cdot\text{mL}^{-1})$	Intra-day ($n = 5$)			Inter-day ($n = 15$)		
	Measured $(\mu\text{g}\cdot\text{mL}^{-1})$	Accuracy/%	Precision (RSD,%)	Measured $(\mu\text{g}\cdot\text{mL}^{-1})$	Accuracy/%	Precision (RSD,%)
RES method						
3'-HP						
0.20	0.21 \pm 0.03	3.31	2.31	0.21 \pm 0.01	5.35	3.68
2.55	2.63 \pm 0.14	3.28	2.28	2.47 \pm 0.13	7.57	5.16
10.19	9.85 \pm 0.15	-3.36	2.42	10.38 \pm 0.89	1.87	1.31
Puerarin						
0.23	0.19 \pm 0.12	-13.83	13.63	0.20 \pm 0.01	-12.22	9.20
2.27	2.56 \pm 0.23	13.13	8.71	2.43 \pm 0.14	7.34	5.01
11.33	12.11 \pm 0.31	6.87	4.69	11.57 \pm 0.62	2.10	1.47
3'-MP						
0.19	0.18 \pm 0.02	-3.41	2.45	0.17 \pm 0.01	-7.71	5.67
2.32	2.62 \pm 0.07	12.86	8.54	2.58 \pm 0.08	11.17	7.48
9.27	9.12 \pm 0.18	-1.67	1.20	9.02 \pm 0.08	-2.70	1.93
DAG						
0.17	0.17 \pm 0.02	3.51	2.44	0.17 \pm 0.01	3.18	2.21
2.08	2.23 \pm 0.08	6.78	4.64	2.22 \pm 0.01	6.54	4.48
8.34	8.17 \pm 0.16	-1.94	1.39	8.12 \pm 0.10	-2.63	1.88
SS method						
3'-HP						
0.20	0.18 \pm 0.01	-12.49	9.42	0.18 \pm 0.01	-9.72	7.23
2.50	2.39 \pm 0.13	-4.32	3.12	2.43 \pm 0.07	-2.79	2.00
10.00	9.72 \pm 0.43	-2.78	2.00	10.05 \pm 0.30	0.48	0.34
Puerarin						
0.19	0.16 \pm 0.01	-13.72	10.41	0.17 \pm 0.01	-10.63	7.94
2.37	2.47 \pm 0.08	4.27	2.96	2.60 \pm 0.12	9.85	6.63
9.48	9.34 \pm 0.49	-1.53	1.09	9.69 \pm 0.37	2.19	1.53
3'-MP						
0.17	0.16 \pm 0.01	-6.49	4.74	0.16 \pm 0.01	-5.61	4.08
2.10	2.30 \pm 0.07	9.41	6.36	2.29 \pm 0.02	9.06	6.13
8.40	8.24 \pm 0.28	-1.95	1.39	8.35 \pm 0.20	-0.57	0.41
DAG						
0.16	0.15 \pm 0.02	-4.48	3.24	0.14 \pm 0.01	-10.80	8.07
1.97	2.04 \pm 0.06	3.42	2.38	2.03 \pm 0.01	2.85	1.99
7.88	7.65 \pm 0.20	-2.89	2.07	7.87 \pm 0.22	-0.10	0.07

Table 4Extract recovery rate and matrix effect for four analytes in dog plasma using RES and SS methods (mean \pm SD, $n = 5$).

Compounds	RES method			SS method		
	Spiked $(\mu\text{g}\cdot\text{mL}^{-1})$	Extract recovery /%	Matrix effect /%	Spiked $(\mu\text{g}\cdot\text{mL}^{-1})$	Extract recovery /%	Matrix effect /%
3'-HP	0.20	78.36 \pm 3.35	100.34 \pm 5.22	0.20	81.78 \pm 5.00	99.66 \pm 3.56
	2.55	74.46 \pm 2.31	89.43 \pm 5.21	2.50	70.67 \pm 4.95	90.12 \pm 3.89
	10.19	79.06 \pm 2.43	90.34 \pm 3.24	10.00	82.24 \pm 3.20	103.79 \pm 7.65
Puerarin	0.23	79.16 \pm 1.74	101.54 \pm 6.54	0.19	80.51 \pm 5.03	101.76 \pm 7.48
	2.27	72.89 \pm 5.30	93.67 \pm 4.43	2.37	73.60 \pm 5.09	95.34 \pm 2.45
	11.33	78.21 \pm 1.46	104.34 \pm 7.23	9.48	74.95 \pm 4.53	101.65 \pm 2.87
3'-MP	0.19	80.72 \pm 1.22	103.45 \pm 6.53	0.17	80.69 \pm 4.47	101.23 \pm 2.34
	2.32	76.84 \pm 3.35	97.43 \pm 5.34	2.10	76.43 \pm 5.59	94.46 \pm 7.64
	9.27	78.44 \pm 5.70	98.34 \pm 6.73	8.40	82.25 \pm 6.74	93.43 \pm 3.23
DAG	0.17	77.72 \pm 2.67	99.76 \pm 5.46	0.16	78.91 \pm 4.06	103.85 \pm 2.37
	2.08	74.70 \pm 3.47	100.29 \pm 2.76	1.97	72.71 \pm 4.80	97.76 \pm 4.87
	8.34	75.35 \pm 1.03	93.46 \pm 8.36	7.88	79.69 \pm 5.94	91.56 \pm 4.50
Liquiritin	0.77	84.07 \pm 1.76	89.35 \pm 6.45	0.77	83.84 \pm 4.34	92.22 \pm 3.54

area under the curve (AUC_{0-t}) was puerarin $>$ 3'-MP $>$ DAG $>$ 3'-HP, which corresponded to the administration dosages except 3'-MP. Although there was no significant difference between the administration dosages of 3'-MP and DAG, there was a significant difference in the AUC_{0-t} values (3'-MP $>$ DAG). In addition, C_{max} for 3'-MP was greater than that for DAG, which indicates that *in vivo* 3'-MP was more strongly absorbed than DAG. The puerarin from the YNT achieved maximum plasma concentration [(940 \pm 185) ng/mL] within (2 \pm 1) h and had the longest retention time [(5 \pm 1) h; Fig. 3, Table 6] demonstrating slow absorption from the gastrointestinal tract and a single absorption peak. These results were consistent with a previous study (Wang, Yao, An, You, & Wang, 2009). The other constituents exhibited similar tendencies. The similar pharmacokinetic behavior of all four flavonoids may be the result of their similar structures. These

results improve our understanding of the therapeutic process of YNT.

Our pharmacokinetic study suggests that similar isoflavones undergo similar processes within a dog's body, and that of the compounds we studied, puerarin had the highest blood concentration and longest retention time in the plasma. These results should be helpful for further studies of the clinical applications of YNT.

The RSD of the mean plasma concentrations of 3'-HP, puerarin, 3'-MP, and DAG calculated by the two methods was less than 5%, and $\cos(\theta) = 1.000$, which indicates a high degree of similarity between results from the quantitative methods of RES and SS. The HPLC chromatograms of pueraria RES and SS exhibited the same retention time and tendencies in Fig. 2. It shows that the RES method is reliable and produces results consistent with the SS method.

Table 5

Pharmacokinetic parameters for four pueraria isoflavones determined following single-dose oral administration of YNT to dogs (130.9 mg/kg, $n = 5$).

Compounds	C_{max} ($\mu\text{g}\cdot\text{mL}^{-1}$)	T_{max} /h	$t_{1/2}$ /h	AUC_{0-t} ($\text{ng}\cdot\text{h}\cdot\text{mL}^{-1}$)	$AUC_{0-\infty}$ ($\text{ng}\cdot\text{h}\cdot\text{mL}^{-1}$)	MRT_{0-t} /h
3'-HP	148.12 \pm 31.21	1.28 \pm 0.86	1.96 \pm 0.47	351.56 \pm 59.98	526.62 \pm 72.92	1.84 \pm 0.40
puerarin	939.97 \pm 185.18	1.80 \pm 0.81	4.22 \pm 1.16	5263.02 \pm 884.88	5611.90 \pm 892.58	5.27 \pm 1.16
3'-MP	255.54 \pm 50.12	2.00 \pm 0.95	3.55 \pm 1.22	1156.96 \pm 117.46 ^a	1600.98 \pm 379.39	3.55 \pm 0.25
DAG	186.36 \pm 42.36	1.97 \pm 1.00	2.54 \pm 0.74	768.07 \pm 98.92	912.20 \pm 40.27	3.16 \pm 0.38

^a $P < 0.05$ (3'-MP vs DAG).

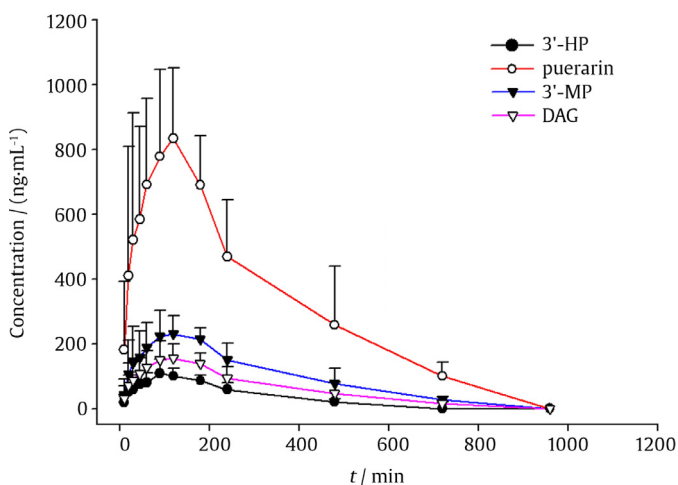


Fig. 3. Mean concentration-time profiles of analytes.

With comparing the SS method, the advantages of RES method are as followed: Firstly, pueraria extract is a multi-component mixture, which can be retained in the proportion of the original medicinal materials, the compound of RES is stable; Secondly, preparation process of the RES method is simple, furthermore, compared with the SS method, the RES method can save the preparation cost; Thirdly, the RES method are low error, simple and convenient preparation.

5. Conclusions

In the present study, the RES method was used to simultaneously quantify the concentrations of several isoflavones from YNT in beagle plasma. This method was characterized by a similar sensitivity, accuracy, reliability, precision, and calibration to the more well-established SS method. The new method is thus a reliable way to simultaneously determine 3'-HP, puerarin, 3'-MP and DAG levels in dog plasma samples since the data were highly consistent with those derived from the SS method. Our results confirm that the RES method can be used for the analysis of biological samples as an alternative to the SS method, to overcome the problem of scarcity of SS.

Declaration of Competing Interest

The authors declare no conflict of interest.

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