

Document heading doi: 10.1016/S1995-7645(14)60334-5

## Pharmacokinetic and pharmacodynamic analysis of ferulic acid–puerarin–astragaloside in combination with neuroprotective in cerebral ischemia/reperfusion injury in rats

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### ARTICLE INFO

#### Article history:

Received 15 January 2015

Received in revised form 20 February 2015

Accepted 15 March 2015

Available online 20 April 2015

#### Keywords:

Ferulic acid

Puerarin

Astragaloside

Interleukin-1  $\beta$

Neuropeptide Y

### ABSTRACT

**Objective:** To investigate the effects of the active ingredients combined therapy on inflammatory factors interleukin 1 beta (IL-1  $\beta$ ) and neuropeptide Y (NPY) based on pharmacodynamics in rats. **Methods:** The animal model was built by transient middle cerebral artery occlusion (MCAO). The method for evaluating the concentrations of the FA-Pr-Al components in rat plasma was established by using HPLC and the expression levels of IL-1  $\beta$  and NPY were determined by ELISA. A new mathematics method of the trend of percentage rate of change (PRC) was used to assess the correlation between pharmacokinetics (PK) and pharmacodynamics (PD). **Results:** FA-Pr-Al in combination reduced neurological deficits, decreased infarct volume and inhibited the expression levels of IL-1  $\beta$  and NPY (all  $P < 0.05$ ) compared with the model group. FA, Pr and Al all displayed two compartment open models in rats. Clockwise hysteresis loops were obtained by time-concentration-effect curves. IL-1  $\beta$  and NPY level changes in the plasma followed an opposite trend to the plasma concentration tendency after  $C_{max}$  was reached. Astragaloside's PRC value was significantly higher than those of FA and puerarin between 120 to 180 min. **Conclusions:** The pharmacokinetics of FA-Pr-Al in combination were closely related its pharmacodynamics in treating ischemia/reperfusion injury, and the components of FA-Pr-Al may have a synergistic pharmacological effect. Astragaloside may play a more pronounced role in regulating IL-1  $\beta$  and NPY levels compared with puerarin or FA.

## 1. Introduction

Cerebral ischemia/reperfusion (I/R) injury during stroke is the third leading cause of death worldwide with a mortality rate approaching 30% and a major cause of disability[1]. Much of the damage caused

by cerebral IR injury is due to inflammatory processes affecting adjacent endothelial cell, neurons, and astrocytes, though the mechanisms associated with such inflammation have recently been characterized. In addition, increased plasma levels of neuropeptides (NP) following ischemic periods were shown to cause thrombosis or cerebral infarction. Inflammation may trigger leucocytes to release proteolytic enzymes, oxygen radicals, and active factors that alter TNF- $\alpha$  and IL-1  $\beta$  levels[2]. Thus, it is an urgent need to develop advanced treatments for inflammation in affected patients.

Ferulic acid (FA), Puerarin(Pr) and Astragaloside(Al) are effective components in many natural medicine, and they were commonly

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Foundation project: This study was supported by the National Science Foundation of China (81274176), the Clinical Medicine Special Foundation of China (L2012064), the National Science Foundation of Province (LY13H280008), and the Science and Technology Department of public welfare project (2014C33212).

used for treating I/R injury in the clinical[3]. Due to the complex chemical composition of natural medicine, classical quality control measures and characterization methods (pharmacokinetic/pharmacodynamic, PK/PD) applied to purified chemical drugs were often not suited for the[4]. How to determine the effective components' action has become an important factor hindering the development of natural products[5]. Multiple studies have been recently focused on correlations between PK/PD and dose, concentration and time, which not only describe and predict the effects of a drug, but also can explore the mechanism of action of effective drugs[6].

The current study aimed to assess the correlation between pharmacokinetics of FA-Pr-Al components and levels of the inflammatory factors IL-1 $\beta$  and neuropeptide Y (NPY) with a new mathematical analysis in the I/R rat model induced by transient middle cerebral artery occlusion (MCAO).

## 2. Materials and methods

### 2.1. Animals

Forty adult male Sprague-Dawley rats weighing ( $280 \pm 20$ ) g were purchased from the Animal Center of Zhejiang University (China, laboratory animal certificate: scxk 2008-0115). Animal care and surgical procedures were performed in accordance with National Institute of Health Guidelines (1996; NIH Publication No. 80-23).

### 2.2. Focal cerebral ischemia–reperfusion rat model induced by MCAO

Rats were randomly subdivided in sham group (negative control), cerebral I/R damage group (model), cerebral I/R damage with FA-Pr-Al treatment (treatment group) and cerebral I/R damage with nimodipine treatment group (positive group) by 10 rats in each group. At a given time point, rats were anesthetized by intraperitoneal (*ip.*) injection of 10% chloral hydrate (0.4 mL/100 g body weight). Cerebral I/R was induced by MCAO using intraluminal filament threads. After maintaining 60-min right MCAO (ischemia), MCA blood flow was restored by withdrawal of nylon sutures[7]. Sham treatment was performed in the same way but without inserting threads. Dead rats and the rats if determined as skull base hemorrhage by autopsy were excluded from the experiment.

### 2.3. FA–Pr–Al treatment

Ferulic acid (Lot No.11773-201211), puerarin (Lot No.130752-201209), astragaloside IV (Lot No. 140781-201113) and nimodipine

(Lot No.18659-201207) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. The ratio of components of FA-Pr-Al was 1:64:28 according to clinical dose equivalent conversion, and then diluted with normal saline into 2.5 g/mL. Rats in the IR+FA-Pr-Al group were orally administered with single-dose of 4.0 g/kg within 30 s after MCAO, while the dose of IR+ nimodipine group was 10 mg/kg. Sham control and I/R model animals were treated each with an equivalent volume of normal saline.

### 2.4. Pharmacokinetic studies

Blood samples (about 500  $\mu$  L at every time) collected via the carotid artery at 0, 5, 15, 30, 60, 120, 180, 240 and 360 min after FA-Pr-Al administration was mixed with 4% trisodium citrate (1:9) and then centrifugated 15 min at 5 000 rpm. The samples were used to detect drug concentration by the methods of HPLC, and to detect the expression of IL-1 $\beta$  and NPY by the methods of ELISA. Pharmacokinetics parameters of the FA-Pr-Al components were computed using the DAS 2.0 software (Chinese Pharmacologic Society, Beijing, China)[8].

### 2.5. HPLC analysis of FA–Pr–Al constituents

HPLC separation was performed on an Agilent 1200 system (Agilent Technologies, USA). FA and Pr were detected using Agilent TC-C18 chromatography column (4.6 mm $\times$ 150 mm; 5  $\mu$  m); the mobile phase was 3% methanol with 3% acetic acid (32:68, v/v) mixture, used at a flow rate of 1.0 mL/min at 25  $^{\circ}$ C. The injection volume was 10  $\mu$  L, and detection was made at 323 nm and 250 nm, respectively. Al was detected by HPLC with Alltech3300 ELSD detector, on Zorbax SB-C18 chromatography column (250 mm $\times$ 4.6 mm; 5  $\mu$  m). The mobile phase was acetonitrile: water (33:67, v/v) used in a drift tube at 100  $^{\circ}$ C (1.0 mL/min flow rate) with 2.0 L/min carrier gas (Pei *et al.*, 2011). 7-hydroxyl coumarin (Lot No. 121739-201001), P-hydroxybenzoic acid (Lot No. 110648-201018), and digoxin (Lot No. 100080- 201006) were used as internal standards for FA, Pr, and Al, respectively. The results were analyzed by the SPSS 11.5 statistical software. Validation of the HPLC assay involved assessing linearity, sensitivity, precision, accuracy, and precision profiles of FA, Pr and Al under various conditions. Selectivity of elution conditions was examined by comparing chromatograms of blank rat plasma from six different rats. Sensitivity was represented by lower limit of quantification (LLOQ), defined as a signal to noise ratio of 3:1. Linear regression was analyzed using GraphPad Prism 5, version 5.03 for Windows (GraphPad Software, San Diego, CA, USA) by the least sum-of-squares method.

## 2.6. Determination of IL-1 $\beta$ and NPY plasma levels

The levels of IL-1 $\beta$  and NPY in rats' plasma were analyzed on an automatic enzyme immunoassay instrument (Hamilton Bonaduz AG, Model F.A.M.E.16/30, Switzerland) using ELISA kits (Santa Cruz Biotechnology Inc., USA). Guided by the manufacturer's protocol, the detection wavelength was at 450 nm.

## 2.7. Pharmacokinetics and pharmacodynamics correlation analysis

Compared with single component drugs, more complexity is expected for the pharmacokinetics and pharmacodynamics of FA-Pr-AI. To elucidate the correlation of pharmacokinetics and pharmacodynamics, the trend chart of Percentage Rate of Change (PRC%) was drawn according to the following formula with  $PRC\% = (T2-T1)/T1 \times 100\%$ , at appropriate intervals between two pharmacokinetic and pharmacodynamic points. Time-concentration-effect curves for FA, Pr, and AI were established with MATLAB 7.0 software (MathWorks, New Mexico, USA). The correlation of pharmacokinetics and pharmacodynamics were analyzed by both trend chart and Time-concentration-effect curves, based on the same type of compartment model with FA-Pr-AI components.

## 2.8. Neurological evaluation

Neurological deficits were evaluated 24 h after reperfusion. Neurologic findings were scored on a 5-point scale (0: no deficit; 1: mild deficit-failure of left forepaw extension; 2: moderate deficit-circling to the left; 3: severe deficit-falling to the left; or 4: critical deficit-depressed consciousness, no spontaneous walking).

## 2.9. Histological infarct size determination by triphenyltetrazolium chloride staining

Rats were euthanized by cervical dislocation, and brains were excised; five brain tissue samples for each group were coronally sliced (2 mm thickness) on a freezing microtome and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C in the dark for 30 min to demarcate the infarct area. Rostral and caudal slices were photographed and analyzed using a digital image analysis system (Sigma Scan Pro, SPSS, Chicago, IL, USA) to indirectly estimate infarct area and volume. Infarct volume (%) was determined as follows:

$$\text{Infarct volume (\%)} = (V_{\text{ipsilateral}} - V_{\text{contralateral}}) / V_{\text{contralateral}} \times 100\%$$

## 2.10. Statistical analysis

All data were expressed as mean  $\pm$  standard deviation (SD).

Statistical analyses were performed with the SPSS 11.5 statistical software. One-way analysis of variance (ANOVA) and LSD *post hoc* methods were used to determine the significance of data sets. *P* values < 0.05 were considered statistically significant.

## 3. Results

### 3.1. Neurological deficits

More severe neurological deficits were observed in the model group compared with animals of the sham group at 24 h. The scores of neurological deficits in animals treated with FA-Pr-AI or nimodipine exhibited significantly less when compared with the model group (Table 1).

**Table 1**

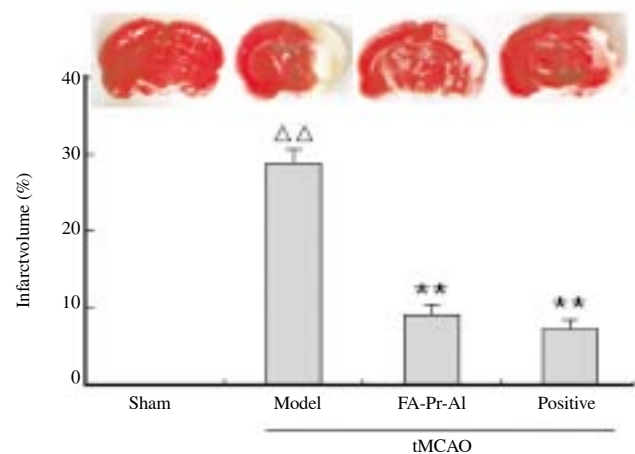
FA-Pr-AI effects on neurologic deficit at 24 h after reperfusion.

Group	dose	n	Neurologic score
Sham group	NS	10	0.20 $\pm$ 0.18
Model group	NS	7	2.80 $\pm$ 0.15 <sup><math>\Delta\Delta</math></sup>
FA-Pr-AI group	4 g/kg	8	1.50 $\pm$ 0.20 <sup>**<math>\Delta\Delta</math></sup>
Nimodipine group	10 mg/kg	8	1.40 $\pm$ 0.27 <sup>**<math>\Delta\Delta</math></sup>

<sup>$\Delta\Delta$</sup>  *P* < 0.01 vs. sham group; <sup>\*\*</sup> *P* < 0.01 vs. model group.

### 3.2. Infarct volume

Infarct volume was significantly greater in the model group compared with sham animals (*P* < 0.01). Quite different with the model group, infarct volumes in FA-Pr-AI treatment rats exhibited significantly decreased, and the similar result were observed from the positive control group (Figure 1).



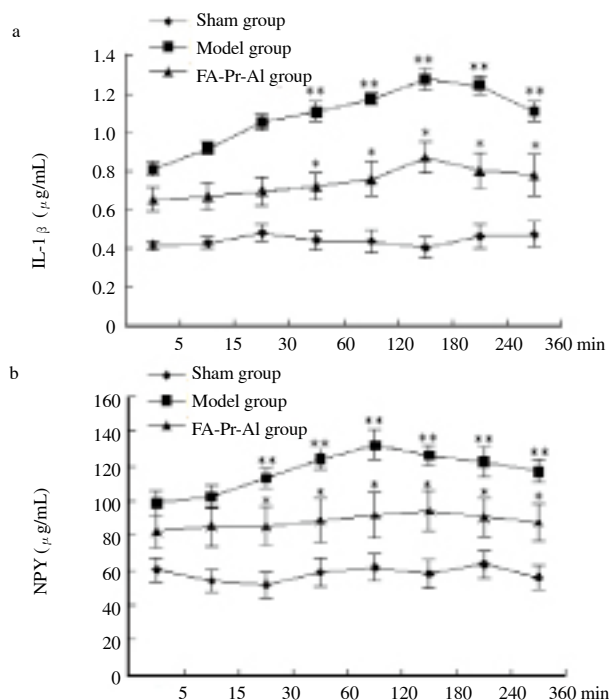
**Figure 1.** Effects of FA-Pr-AI on infarct volume in a rat model of cerebral ischemia/reperfusion (I/R).

TTC leaves infarcted tissue unstained (white), while viable tissue is stained (red).

<sup>$\Delta$</sup>  *P* < 0.05,  <sup>$\Delta\Delta$</sup>  *P* < 0.01 vs. sham group; <sup>\*</sup> *P* < 0.05, <sup>\*\*</sup> *P* < 0.01 vs. model group. Positive control: Nimodipine group.

### 3.3. IL-1β and NPY levels

The expression levels of IL-1β and NPY were increased in model group rats' plasma compared with sham animals at all time points (all  $P < 0.01$ ), while the levels in FA-Pr-AI treatment group were decreased significantly at 60, 120, 180, 240, and 360 min (all  $P < 0.01$ ; Figure 2).



**Figure 2.** Effects of FA-Pr-AI on plasma NPY and IL-1β levels in a rat model of I/R. NPY and IL-1β levels were determined by ELISA. (A) Plasma IL-1β levels. (B) Plasma NPY levels.  $^{\Delta} P < 0.05$ ,  $^{\Delta\Delta} P < 0.01$  vs. sham group; \* $P < 0.05$ , \*\* $P < 0.01$  vs. model group. IL-1β: Interleukin-1 beta; NPY: Neuropeptide Y.

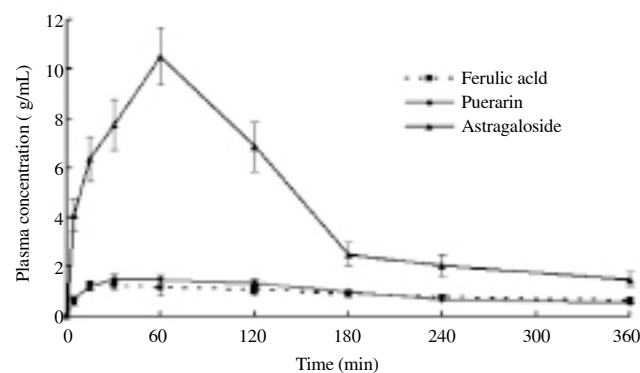
### 3.4. Chromatographic separation and determination of FA-Pr-AI components

Typical chromatograms of FA demonstrated an average recovery ( $n=6$ ) of 96.41% (RSD=2.36%), with FA and 7-hydroxyl coumarin

peaks clearly separated, with retention times of 6.6 and 5.8 min, respectively. Meanwhile, typical chromatograms of puerarin demonstrated an average recovery ( $n=6$ ) of 94.84% (RSD=3.63%), with puerarin and p-hydroxybenzoic acid peaks clearly separated, with retention times about 7.2 and 5.5 min, respectively. Typical chromatograms of astragaloside demonstrated an average recovery ( $n=6$ ) of 90.54% (RSD=5.05%), denoting clearly that separated astragaloside and digoxin peaks with retention times was about 8.2 and 3.8 min, respectively.

### 3.5. Pharmacokinetic parameter determination of FA-Pr-AI components

Pharmacokinetic parameters of ferulic acid, puerarin and astragaloside were summarized in (Table 2). The pharmacokinetic profiles of the three compounds (FA, Puerarin and Astragaloside) administered orally displayed typical bi-exponential decline; their plasma concentration-time data were fitted into classical two-compartment first-order open model ( $C = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$ ) using a weighting factor of  $1/y^2$  which can make them have the same type of metabolism in rats after a single oral administration of FA-Pr-AI (4.0 g/kg) (Figure 3).



**Figure 3.** Plasma concentrations of the ferulic acid, puerarin, and astragaloside over time.

Plasma samples were harvested from rats in each group at the time points indicated. The concentrations of components were determined by HPLC. The mean  $\pm$  SD was shown for each time point.

**Table 2**

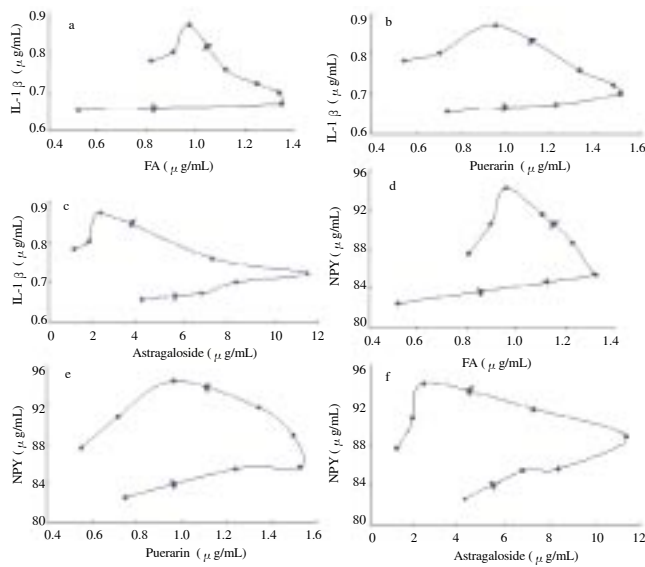
Pharmacokinetic parameters of the ferulic acid, puerarin and astragaloside in rats after oral administration of FA-Pr-AI (4 g/kg) after MCAO (means  $\pm$  SD) ( $n=6$ ).

Parameter	Ferulic acid	Puerarin	Astragaloside
AUC <sub>(0-t)</sub> (min×mg/L)	405.4±60.1	422.6±64.4	1 741.2±145.6
AUC <sub>(0-∞)</sub> (min×mg/L)	775.8±59.4	795.0±127.5	1 977.1±153.2
t <sub>1/2z</sub> (min)	333.2±79.8	510.7±48.6	104.5±2.6
CLz/F (L/min/kg)	0.026±0.001	0.017±0.002	0.010±0.001
Vz/F (L/kg)	12.4±3.3	12.9±3.1	1.5±0.2
C <sub>max</sub> (mg/L)	1.6±0.3	1.6±0.1	11.0±0.5
T <sub>max</sub> (min)	15	30	60

Notes: AUC was determined as area under the concentration versus time curve; C<sub>max</sub>, maximal concentration; T<sub>max</sub> was determined as the time when the maximal concentration reached; CLz/F, clearance; Vz/F, apparent volume of distribution; t<sub>1/2z</sub>; terminal half-life.

### 3.6. Time–concentration–effect curves

At the time points, concentration and effect did not display a strict one-to-one correspondence after oral administration. One plasma concentration of FA-Pr-Al corresponded to two kinds of efficacy, and maximal effects were lagged behind peaks of plasma concentration. Notably, the time-concentration-effect curves were anticlockwise hysteresis loops between the plasma concentration of Ferulic acid and the levels of IL-1  $\beta$  and NPY. However, clockwise hysteresis loops were obtained for time-concentration-effect curves between puerarin and astragaloside plasma concentrations and the levels of IL-1  $\beta$  and NPY (Figure 4).

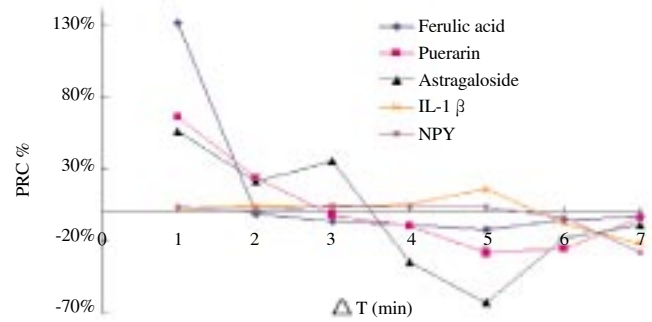


**Figure 4.** Relationship between plasma concentrations of the ferulic acid, puerarin, and astragaloside, and plasma efficacy by IL-1  $\beta$  and NPY levels.

Arrows indicate the direction of time. (a) plasma FA and IL-1  $\beta$  contents; (b) plasma puerarin and IL-1  $\beta$  contents; (c) plasma astragaloside and IL-1  $\beta$  contents; (d) plasma FA and NPY contents; (e) plasma puerarin and NPY contents; (f) plasma astragaloside and NPY contents.

### 3.7. Pharmacokinetic and pharmacodynamic correlation analysis

Compared with the PRC values for IL-1  $\beta$  and NPY plasma levels, the plasma concentration change tendency in PRC of FA-Pr-Al components were the opposite direction after the time corresponding to the  $C_{max}$ . The PRC value obtained for astragaloside was significantly higher than what observed for FA and Pr at the fifth point of time, between 120 to 180 min. These results suggested that astragaloside may play a more pronounced role in regulating IL-1  $\beta$  and NPY levels, compared with puerarin or FA (Figure 5).



**Figure 5.** PRC(%) between two pharmacokinetic or pharmacodynamic points.

The PRC was derived with the formula of  $(T_2 - T_1)/T_1 \times 100\%$ .  $\Delta T_1$  represents two points from 5 min to 15 min of pharmacokinetics or pharmacodynamics respectively.  $\Delta T_2$  represents from 15 min to 30 min.  $\Delta T_3$  represents from 30 min to 60 min.  $\Delta T_4$  represents from 60 min to 120 min.  $\Delta T_5$  represents from 120 min to 180 min.  $\Delta T_6$  represents from 180 min to 240 min.  $\Delta T_7$  represents from 240 min to 360 min.

## 4. Discussion

Several nature medicine products have been shown to exert protective effects against cerebral ischemia-reperfusion injury such as Salvianolate, hydroxy Safflower Pigment A, breviscapin, and ligustrazine. These products achieved neuroprotection through various mechanisms, including upregulating the expression of heat shock protein 22, phosphorylated protein kinase B, transforming growth factor-  $\beta$  1, and GM130 as well as activation of PI3K/Akt and the Nrf2 signaling pathway[10].

In this study, we demonstrated that animals treated with FA-Pr-Al exhibited significantly less severe neurological deficits and decreased infarct volumes. At the molecular, these effects were accompanied by markedly decreased IL-1  $\beta$  and NPY, indicating a neuroprotective effect for FA-Pr-Al after I/R injury.

Next, we obtained blood drug concentration of FA, Pr and Al in FA-Pr-Al in combination as well as IL-1  $\beta$  and NPY in rats at different time points by pharmacokinetics and pharmacodynamics. However, the results of the time-concentration-effect curves and compartmental models suggested that FA-Pr-Al may affect not only local cerebral tissues, but also a wide variety of systemic organs and tissues. Because of time lag of blood drug concentration, it is difficult to directly analyze the relationship between the pharmacokinetics and pharmacodynamics[11].

We have studied the correlation of pharmacokinetic and pharmacodynamic on FA-Pr-Al, based on the analysis of the change tendency in PRC values for plasma FA-Pr-Al component concentrations as well as plasma IL-1  $\beta$  and NPY levels, so as to determine the effect of individual components on drug pharmacodynamics. PRC represents the metabolic rate of change



over a period of time. This formula of PRC is a new mathematical analysis method, which allows a more intuitive description of the correlation of PD/PK and helps us to determine the components that affect certain pharmacodynamic parameters from the cure graph. From the trend chart the astragaloside metabolic rate decreased at a more pronounced level compared with FA and puerarin after blood concentration peaked, while pharmacodynamics of IL-1  $\beta$  and NPY levels showed negligible changes in rats at the same time. It is obvious that the opposite tendency between the changes of blood FA-Pr-Al component concentration correlates with efficacy, especially, the change of PRC from the third to sixth value for FA, and the change of PRC from the fourth to sixth value for puerarin and astragaloside. These findings indicate the fast nature medicine metabolism and its obvious effects. According to the trend chart, it appears that the three components in FA-Pr-Al may play a synergistic pharmacodynamic effect. Indeed, emerging evidence has suggested that multiple compounds from herbals might demonstrate synergism. The concentration changes of astragaloside noticeably accelerated from the fourth to sixth value, while the change in IL-1  $\beta$  levels was also evident. These results suggest that IL-1  $\beta$  may be a target of astragaloside or the latter may play a major role in IL-1  $\beta$  homeostasis. Nevertheless, the change on the levels of NPY was not significant, suggesting that NPY may not a target of astragaloside, FA or puerarin.

This research was completed according to the “nature medicine indications Pharmacokinetics” guiding principles[12]. The idea was to identify the role of the compound in vivo and the metabolic mechanisms associated with the drug ingredients. This has been done by quantitatively describing the composition and efficacy in combination with the appropriate mathematical methods for assessing the relationship between them and the existing law. This mathematical method for PK-PD analysis allowed the identification of high correlation ingredients, thus the pharmaceutical ingredients obtained may be regarded as “indications of ingredients.” Furthermore, this mathematical method used for PK / PD analysis may also strongly correlate with unknown components, suggesting that the medicine may have other not yet detected component with strong pharmacological activity.

In FA-Pr-Al, the diverse components may have a synergistic pharmacological effect, which may be typical of nature medicine and its merits will be further investigated. Furthermore, astragaloside may play a more pronounced role in regulating IL-1  $\beta$  and NPY levels compared with puerarin or FA. The mathematical method can be used to find a strong correlation ingredient for PK-PD analysis.

### Conflict of interests statement

We declare that we have no conflict of interests.

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