Pharmacokinetic-Pharmacodynamic Analysis on Inflammation Rat Model after Oral Administration of Huang Lian Jie Du Decoction

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Abstract

Huang-Lian-Jie-Du Decoction (HLJDD) is a classical Traditional Chinese Medicine (TCM) formula with heat-dissipating and detoxifying effects. It is used to treat inflammation-associated diseases. However, no systematic pharmacokinetic (PK) and pharmacodynamic (PD) data concerning the activity of HLJDD under inflammatory conditions is available to date. In the present study, the concentration-time profiles and the hepatic clearance rates (HCR) of 41 major components in rat plasma in response to the oral administration of a clinical dose of HLJDD were investigated by LC-QqQ-MS using a dynamic multiple reaction monitoring (DMRM) method. Additionally, the levels of 7 cytokines (CKs) in the plasma and the body temperature of rats were analyzed. Furthermore, a PK-PD model was established to describe the time course of the hemodynamic and anti-inflammatory effects of HLJDD. As one of the three major active constituents in HLJDD, iridoids were absorbed and eliminated more easily and quickly than alkaloids and flavonoids. Compared with the normal controls, the flavonoids, alkaloids and iridoids in inflamed rats exhibited consistently changing trends of PK behaviors, such as higher bioavailability, slower elimination, delays in reaching the maximum concentration (T_{max}) and longer substantivity. The HCR of iridoids was different from that of alkaloids and flavonoids in inflamed rats. Furthermore, excellent pharmacodynamic effects of HLJDD were observed in inflamed rats. The levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β, IL-10, and macrophage inflammatory protein-2 (MIP-2) and body temperature significantly decreased after the administration of HLJDD. Based on PK-PD modeling with the three-phase synchronous characterization of time-concentration-effect, flavonoids exhibited one mechanism of action in the anti-inflammatory process,

while iridoids and alkaloids showed another mechanism of action. Taken together, the results demonstrated that HLJDD may restrain inflammation synergistically via its major constituents (alkaloids, flavonoids and iridoids). A correlation between the exposure concentration of different types of compounds and their anti-inflammatory effects in the body was shown. This study provides a comprehensive understanding of the anti-inflammatory activity of HLJDD.

Introduction

Huang-Lian-Jie-Du Decoction (HLJDD) is a classical Traditional Chinese Medicine (TCM) formula with heat-dissipating and detoxifying effects. It is used to treat inflammation-associated diseases. It is comprised of *Rhizoma coptidis*, *Radix scutellariae*, *Cortex phellodendri* and *Fructus gardenia* at a ratio of 3:2:2:3. HLJDD exerts various bioactivities on multiple types of tumors [1–5], arthritis [6–8], polymicrobial sepsis [9], cardiac damage [10], liver injuries [11], type II diabetes [12–14], and Alzheimer's disease [15]. These effects are closely associated with its anti-inflammatory activity, which is synergistically exhibited by the major ingredients in HLJDD [16].

Alkaloids, flavonoids and iridoids were reported to be the main bioactive constituents in HLJDD [16–18], and they individually act on specific targets in the inflammatory process [16, 19, 20]. Their preliminary pharmacokinetic behaviors have been studied in recent years [21–25]. However, only six constituents were examined in previous studies, which did not fully illustrate the complete plasma pharmacochemistry profile of HLJDD [21]. Additionally, it is difficult to obtain meaningful information on the efficacy and exposure concentration *in vivo* [23]. This unsatisfactory level of data can be attributed to imprecise experimental instruments, such as HPLC, which presents low sensitivity, poor selectivity and a limited ability to detect active components. In contrast, UHPLC combined with triple quadruple mass spectrometry (QqQ-MS) exhibits a faster analytical speed, a wider quantification range and higher sensitivity [26, 27]. These advanced features help facilitate the analysis of the formula and plasma profile of complex chemicals, such as TCM [18, 24].

Carrageenan (Ca) and lipopolysaccharide (LPS) have been used to induce inflammation *in vivo* [8, 20, 28, 29]. Some pure compounds isolated from HLJDD had positive effects on inflammation [2, 16, 30–34]. It is well known that separate pharmacodynamic assessments cannot fully describe the complete effect of HLJDD on inflammation. The pharmacokinetic-pharmacodynamic (PK-PD) method, which has been used extensively in drug screening, clinical trial design and the selection of dose regimens [35], is a feasible way to clarify the synergism of a formula's multiple components [36]. In our previous study, the chemical profiling of the main constituents of HLJDD in rat plasma was comprehensively clarified, and 17 representative compounds in the extract of HLJDD were simultaneously quantified as quality control markers [18, 37]. Additionally, the distinctive metabolic processes of three types of representative components in HLJDD were clarified, and the *in vivo* metabolic network of HLJDD was illustrated [38]. These results laid a solid foundation for the PK-PD analysis of HLJDD in a pathological rat model.

In this pharmacokinetic study, we investigated the concentration-time profiles and the hepatic clearance rates (HCR) of 41 major components of HLJDD after the oral administration of a clinical dose in rats. LC-QqQ-MS in the dynamic multiple reaction monitoring (DMRM) mode was used for the PK analysis. Simultaneously, the body temperature and the levels of 7

cytokines (CKs), including TNF-α, IL-6, IL-1β, IFN-γ, IL-10, IL-13 andMIP-2, were estimated. Furthermore, a logistic transition mathematical model was established based on the PK and PD data to investigate the relationship between pharmacokinetic exposure to the active substances in HLJDD and the pharmacodynamic response. This study provided a comprehensive understanding of the anti-inflammatory activity of HLJDD.

Materials and Methods

Materials and Reagents

Radix scutellariae, *Rhizoma coptidis*, *Cortex phellodendri chinensis* and *Fructus gardenia* originated from the same batch as that was used in our previous reports [18, 37, 38]. Reference standards of berberine, baicalin, wogonin, oroxylin A and geniposide were isolated from HLJDD in our laboratory [39]. Magnolflorine was purchased from Beijing Saibaicao Co., Ltd. (Beijing, China). Wogonoside and oroxylin A-7-O-glucuronide were provided by Ze Lang Medical Technologies Co., Ltd. (Nanjing, China). Swertiamarin (IS1), corynoline (IS2) and icariin (IS3) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and employed as the internal standards for iridoids, alkaloids and flavonoids, respectively. Ascorbic acid (batch number: 20120214) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). LPS (derived from *Escherichia coli* 0111: B4) and Ca were obtained from Sigma (St. Louis, MO, USA). HPLC-grade methanol and analytical-grade acetonitrile were purchased from Honeywell Burdick & Jackson (Swedesboro, NJ, USA). Formic acid was obtained from Thermo Fisher Scientific (Bremen, Germany),and ultrapure water was purified by a Millipore system (Millipore, Billerica, MA, USA). Other chemicals and solvents were of analytical grade.

AimPlex™ assay kits, including an AimPlex® Rat custom 7-plex kit (cat number: T311C07) and an AimPlex® Mouse/Rat Basic kit (cat number: R200201), were provided by Beijing Quantobio Biotechnology Co.,Ltd. (Beijing, China).

Plant Extraction and Preparation of Solutions

Four samples of dried and crushed plant material were homogenized at a ratio of 3:2:2:3 (*Coptidis rhizoma*:*Scutellariae radix*:*Phellodendri chinensis cortex*:*Fructus gardeniae*) and decocted twice with boiling water (1:10, w/v) for 2 h. The aqueous extract was concentrated to a constant weight on a rotary vacuum evaporator at 80°C and crushed into powder before the experiment. HLJDD extract powder (HLJDD-EP), Ca and LPS were individually dissolved in saline solution via ultrasonication at a concentration of 35.0 mg/mL for HLJDD-EP, 25.0 mg/mL for Ca and 50.0 μg/mL for LPS. Eight reference standards (geniposide, magnolflorine, baicalin, berberine, oroxylin A-7-O-glucuronide, wogonoside, wogonin and oroxylin A) were dissolved in methanol and diluted to a series of concentrations. An internal standard stock solution (containing swertiamarin, corynoline and icariin) was also prepared with methanol.

Surgical Procedure and Collection of Biosamples

Sprague-Dawley rats (male, 200±20 g), provided by Cisco North Biotechnology Co., Ltd.(Beijing, China), were maintained in polypropylene cages with constant access to rodent chow (Nanjing, China) and water in an environmentally controlled room (12 h light cycle) at 20 ±1°C and 50±10% relative humidity. The rats were acclimatized to the facilities for 5 days and screened via the continuous determination of the rectal temperature for two days. Rats with temperatures over 38°C and those that exhibited a difference between two measured temperatures greater than or equal to 0.5°C were excluded from the experiment. Rats in the inflamed

groups previously received intraperitoneal injections of Ca (0.1 mL/100 g) and were fasted with free access to water for 16 h, followed by intravenous injections of LPS (0.1 mL/100 g) to induce inflammation. The control group G1 was injected with the same volume of physiological saline. The ethics committees of Cisco North Biotechnology Co., Ltd. (Beijing, China) and the China Academy of Chinese Medical Sciences (Beijing, China) approved the experimental protocol. The ethical approval number was BJ-2014-0125-01.

For the PK-PD analysis, cannulas were implanted in each rat's jugular vein (n = 22). To study the hepatic first-pass effect (HFPE), the jugular vein and the pyloric vein of each rat (n = 5) were cannulated according to the method described by Hye J. Chung *et al.* with slight modifications [40]. Instead of the portal vein, the pyloric vein was cannulated to minimize the interference of blood flow in the portal vein [41]. The surgery was performed under anesthesia (3% chloral hydrate solution, intraperitoneal injection; 3.5 mL/kg) 5 days prior to the experiment. Rats with jugular vein intubation surgery were divided into four groups: a control group with HLJDD (G1, n = 6), aninflammatory group with HLJDD (G2, n = 6), aninflammatory group with physiological saline (G3, n = 5) and another inflammatory group with HLJDD (G4, n = 5). G1 and G2 were used for the pharmacokinetic study, while G3 and G4 were used for the pharmacodynamic study. Rats with jugular and pyloric vein cannulation for HFPE analysis were assigned to another inflammatory group with HLJDD (G5, n = 5).

The prepared HLJDD-EP solution was administered to the rats (G1, G2, G4 and G5) at 2 mL/100g body weight (crude material content: 3.5 g/kg) by oral gavage, and the G3 group was given the same volume of physiological saline. In the G1 and G2 groups, blood specimens (300 μL) were obtained before dosing and subsequently at 5 min, 10 min, 30 min, 1, 2, 4, 6, 8, 12, 24, 36 and 48 h after the oral administration of HLJDD-EP solution. In the G3 and G4 groups, blood samples (300 μL) were collected prior to dosing and followed at 10 min, 30 min, 1, 2, 4, 8, 24 and 48 h after drug administration. The rectal temperature of rats in the G3 and G4 groups was immediately monitored after each collection of blood. For the HFPE assay, blood specimens (150 μL) from both the jugular vein and the pyloric vein of the G5 rats were simultaneously taken before dosing and subsequently at 5 min, 10 min, 30 min, 1, 2, 4, 6, 8, 12, 24, 36 and 48 h after oral administration. Samples were collected in heparinized Eppendorf tubes and centrifuged at 3000 rpm for 15 min. The plasma was stored at -80°C until assay.

Determination of the 41 Components of HLJDD in Rat Plasma

For the pharmacokinetic investigation and HFPE analysis, 100 μL aliquots of plasma were mixed with 5 μL of ascorbic acid (dissolved in physiological saline, w/v: 1 g/100 mL) and 200 μL of methanol (containing an internal standard stock solution with 80.0 ng/mL swertiamarin, 1.5 ng/mL corynoline and 33.0 ng/mL icariin), followed by vortexing and centrifuging at 15000 rpm for 20 min to remove the precipitate. The supernatant was dried with nitrogen at 40°C. The residue was re-dissolved in 100 μL of methanol and centrifuged at 15000 rpm for 10 min. The supernatant was analyzed using Agilent 1290 ultra-high performance liquid chromatography coupled with an Agilent 6490 Triple Quadrupole Mass Spectrometer (Agilent Technologies, Palo Alto, CA, USA).

Liquid chromatographic separations of the analytes were performed using a Thermo Scientific Hypersil GOLD column (50 mm× 2.1 mm, 1.9 μm). The mobile phase consisted of acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). The gradient elution was as follows: 0–3 min, linear from 5% to 10% A; 3–10 min, linear from 10% to 23% A; 10–15 min, linear from 23% to 50% A; 15–16 min, linear from 50% to 100% A; 16–17 min, held at 100% A; 17–18 min, linear from 100% to 5% A; and 18–20 min, held at 5% A for equilibration of the column. The flow rate was 0.3 mL/min. The injection volume was 2 μL. The column

temperature was kept at 35°C, and the sampler temperature was set at 4°C. The optimized ESI source parameters were as follows: gas temperature, 200°C; gas flow, 14 L/min; nebulizer pressure, 20 psi; sheath gas temperature, 400°C; and sheath gas flow, 11 L/min. Analytes were quantitated by monitoring the precursor-product combination in the DMRM mode using ion polarity switching (flavonoids and alkaloids were determined in the positive mode, while iridoids were detected in the negative mode). To ensure the desired abundance of each compound, the CE values and other parameters were optimized and illustrated as follows: cycle time, 300 ms; positive capillary voltage, 3000 v; negative capillary voltage, 2000 v; positive nozzle voltage, 1500 v; negative nozzle voltage, 1000 v; Delta EMV(+), 200 v; Delta EMV(-), 200 v. The optimized mass transition ion pairs (m/z) for analytes and the detection of the conditions of the compounds are shown in S1 Table.

The current UHPLC-QqQ-MS method was validated for its linearity, intra-day and inter-day precision, accuracy, stability, extraction recovery and matrix effect. The typical chromatograms of plasma samples showed an ideal signal response, excellent resolution, clear exhibition of shapes and short run time (S1 Fig). The regression equations, correlation coefficients, test ranges and limits of quantification (LOQ) are shown in S2 Table. All the calibration curves showed an excellent correlation between the ratio of peak area and concentrations for each compound within the test ranges. The intra- and inter-day precision of these analytes (relative error, RE) were less than -17.9% and 14.8%, respectively (S3 Table). The 8 analytes in plasma were stable (S4 Table) under the following conditions: 4 h at room temperature, 15 days of storage at -80°C, 3 freeze-thaw cycles at -80°C and 48 h in the autosampler. The extraction recoveries of the 8 standards (S5 Table) were all within the acceptable ranges (from 82.41% to 105.98%), except for baicalin (from 68.52% to 80.20%), and all of the results showed good accuracy and precision. The low extraction recovery of baicalin was in accordance with the published results that its mean recovery in rat plasma ranged from 70.51% to 75.28% [42]. Most of the average matrix effect values of the analytes at 3 quality control (QC) concentrations ranged from 80% and 120%, except for baicalin (ranged from 68.13% to 75.73%), which indicated that no co-eluting unseen compounds significantly influenced the ionization of the analytes.

Simultaneous Measurements of 7 Cytokines in Rat Plasma

The measurement of 7 cytokines, including IFN-γ, IL-6, IL-1β, MIP-2, TNF-α, IL-13 and IL-10 in rat plasma was conducted using a commercially available rat flow cytomix basic kit (BD Biosciences). All of the procedures were performed by the same operator according to the manufacturer's protocol. Standard curves for each biomarker were generated with 7 serial concentrations following the manufacturer's instructions.

Data Analysis

Pharmacokinetic parameters of the determined compounds were processed by the non-compartmental method using Phoenix Winnonlin software (Pharsight Corporation, USA). Linear trapezoidal integration was used to calculate areas under the concentration-time curves (AUC) and areas under the first moment curves (AUMC). The mean residence time (MRT) was determined as AUMC/AUC. The plasma half-life ($HL_{\lambda z}$), maximum plasma concentration (C_{max}), the time to reach C_{max} (T_{max}) and other parameters following the administration of HLJDD were determined from the observed data. The HFPE was defined as HCR: HCR% = $100 \times (AUC_{0-t}$ in the pyloric vein-AUC_{0-t} in the jugular vein)/AUC_{0-t} in the pyloric vein. Concentrations of cytokines were analyzed by cytometric bead array software (FCAP Array version 3.0, BD Bioscience). All the data were expressed as the mean±standard deviation (SD). A p value less than 0.05 was considered statistically significant.

Based on the PK and PD data, a PK-PD fitted mathematic model was established to investigate the relationship between the active substances in HLJDD and the pharmacodynamic indexes. After continuity correction with a logistic steady transformation, the empirical distribution function of data for each time point was calculated. To form virtual sample paths, the bootstrap re-sampling method was used for random data extraction from each time point. Thus, the pharmacokinetic and pharmacodynamic measurement of one rat was emulated. In the present study, the number of re-samplings was set as M = 200. The logistic transformation formula was as follows:

$$R(t) = \frac{1}{1 + \exp\{\beta(x - \alpha)\}}$$

α and β represent the positional parameters and scale parameters for each variable, respectively.

The cumulative effect of each time point was selected as the characteristic index in the analysis. The cumulative index of the control group was utilized as a background to correct the baseline drift. The polynomial interpolation method was used to obtain each value of each time point along the virtual paths. Furthermore, the average effect of the pharmacokinetic indexes on the pharmacodynamic indexes was investigated by establishing a dynamic model of stochastic differential equations (defined below):

$$dY(t)/dX(t) = A(t) + \varepsilon(t)$$

X(t) and Y(t) represent the cumulative effect of the various indexes, and A(t) represents the change rate of the pharmacodynamic indexes with respect to the pharmacokinetic indexes.

The cubic spline method was used to calculate the values of the partial derivative for each virtual efficacy path in terms of the aforementioned 8 components. As a result, the ingredient and effect index of every virtual path at any point in time produced a series of derivative values (M = 200). Then, the density estimation of the partial derivative from the effectiveness index of each ingredient at every time point was obtained by kernel density estimation. If the efficacy of an ingredient was not significant, the probability density of the derivative approached zero; otherwise, it moved to positive or negative areas. Based on their average variation rate, the relationship between the pharmacokinetic and pharmacodynamic indexes was deduced.

Results

Measurement of 41 Components in Rat Plasma

The validated method was successfully applied to the pharmacokinetic and HFPE analysis of 41 compounds in rat plasma. The plasma concentration-time curves (n = 6) of geniposide, magnolflorine, berberine, baicalin, oroxylin A-7-O-glucuronide, wogonoside, wogonin and oroxylin A in normal rats are shown in Fig 1, and their estimated pharmacokinetic parameters are presented in Table 1. The peak area ratio-time curves (n = 6) of the other 33 ingredients are shown in S2 Fig. The results demonstrated that iridoids were absorbed and eliminated more easily and quickly than alkaloids and flavonoids. The T_{max} and MRT_{last} of geniposide were 0.7 ±0.3 h and 0.9±0.1 h, respectively. The pharmacokinetic behaviors of aporphine alkaloids were different from those of protoberberine alkaloids, which may have been caused by differences in their absorption in the gastrointestinal tract. Flavonoids exhibited similar T_{max} and MRT in rat plasma. All of the flavonoids exhibited a bimodal phenomenon in rats, with C_{max1} at 5–10 min and C_{max2} at 8–12 h, which was consistent with previously published reports [22, 43].

Compared to the controls, the pharmacokinetic behaviors, including $HL_{\lambda z}$, T_{max} and AUCs of most flavonoids, alkaloids and iridoids significantly increased in inflamed rats after the oral
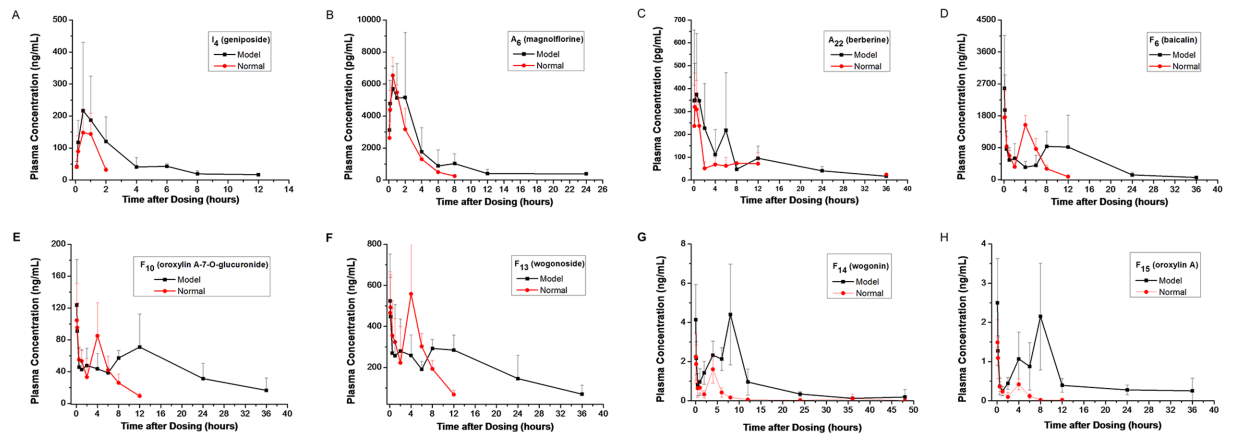
Fig 1. The plasma concentration-time curves of geniposide, magnolflorine, berberine, baicalin, oroxylin A-7-O-glucuronide, wogonoside, wogonin and oroxylin A in normal and inflamed rats.

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administration of the HLJDD extract. It was observed that the AUC_{last} values of geniposide, magnolflorine, berberine, baicalin and wogonin in inflamed rats were 637.6, 24.9, 2.7, 15737.7 and 38.7 h*ng/ml, respectively, while those in normal rats were 216.8, 16.0, 1.3, 7881.1 and 7.2 h*ng/ml, respectively. The mean AUC_{last} values of geniposide, magnolflorine, berberine, baicalin and wogonin plasma concentrations were 2.9-, 1.6-, 2.1-, 2.0- and 5.4-fold larger in the inflamed group than those in the control group.

According to the obtained AUC_{0-t} of the 41 compounds in the plasma obtained from the jugular vein and the pyloric vein of the inflamed rats (Fig 2), the HCR was calculated (Fig 3). The HCR values of I_1 and I_3 were -43.7% and -156.6%, respectively, due to the cross-metabolism of iridoids. Specifically, some iridoids were metabolized into I_1 and I_3 in the liver. It is worth noting that the HCR values of I_2, F_4 and F_7 were approximately zero, indicating that these compounds exhibited good bioavailability after oral administration. In comparison, all of the alkaloids and most of the flavonoids exhibited a relatively higher HCR value.

Pharmacodynamic Analysis of HLJDD

Significant differences in body temperature were found in the inflamed rats before and after modeling. All of the cytokines except IFN-γ increased at a certain time point between normal and model rats (Fig 4).The results suggested that CKs, including IL-6, IL-1β, MIP-2, TNF-α, IL-13 and IL-10, were closely associated with the inflammation induced by LPS and Ca. These findings are consistent with the published literature [44]. Furthermore, levels of IL-6, IL-1β, MIP-2, TNF-α, and IL-10and body temperature were down-regulated after the administration of HLJDD. Specifically, HLJDD may significantly reduce the expression of MIP-2, TNF-α and IL-1β at 2 h, IL-1β at 4 h and IL-10 at 2–8 h in the plasma of inflamed rats and down-regulate the body temperature 8 h after the oral administration of HLJDD (Table 2). The results indicated that HLJDD may prevent an excessive inflammatory response under acute inflammatory conditions.

Pharmacokinetic-Pharmacodynamic Analysis

First, equipotential curves were fabricated based on the probability density matrix to clearly express the change in the rates of pharmacodynamic indexes to pharmacokinetic indexes. For example, from PK1 (baicalin) to PD1 (IL-6) (Fig 5), the average rate of change exhibited greater

Table 1. The pharmacokinetic parameters of geniposide, magnolflorine, baicalin, berberine, oroxylin A-7-O-glucuronide, wogonoside, wogonin and oroxylin A in normal and inflamed rats.

PK parameters	Group	baicalin	oroxylin A-7-O-glucuronide	wogonoside	geniposide	magnolflorine	berberine	wogonin	oroxylin A
Dose (g/kg)	B/M	0.1246	0.0236	0.0910	0.0881	0.0024	0.0296	0.0063	0.0025
$HL_{\lambda z}$ (h)	B	1.8±0.4	3.2±0.9	3.0±1.0	0.6±0.3	1.6±0.4	6.3±3.5	3.5±1.3	1.7±0.9
	M	4.7±1.1	20.1±6.1	12.2±5.9	1.6±0.7	4.3±2.2	9.1±2.5	7.1±2.7	16.3±8.8
Tmax1 (h)	B	0.09±0.03	0.09±0.03	0.11±0.04	0.7±0.3	0.6±0.2	0.6±0.4	0.09±0.03	0.08±0.0
	M	0.1±0.04	0.09±0.03	0.16±0.08	0.8±0.3	0.6±0.3	1.0±0.6	0.08±0.0	0.08±0.0
Tmax2 (h)	B	4.3±0.8	4.0±0.0	4.3±0.8	-	-	8.0±2.2	4.0±0.0	4.0±0.0
	M	9.3±2.1	9.6±2.2	8.7±3.0	-	-	10.8±2.7	7.3±2.7	7.0±3.0
Cmax1 (ng/ml)	B	1828.8±737.4	106.9±43.8	492.9±152.9	171.6±70.6	6.5±1.1	0.4±0.1	2.2±1.2	1.5±0.6
	M	2420.9±922.5	108.7±47.6	571.9±183.4	223.2±43.7	6.0±1.7	0.5±0.2	3.3±0.7	2.5±1.1
Cmax2 (ng/ml)	B	1580.1±202.4	85.8±40.3	517.7±173.8	-	-	0.1±0.03	1.6±0.7	0.4±0.1
	M	1000.4±298.1	65.5±18.2	323.9±52.5	-	-	0.3±0.3	4.3±1.8	2.3±0.8
AUC_{last} (h*ng/ml)	B	7881.1±774.7	440.7±106.9	3302.3±975.1	216.8±86.2	16.0±2.0	1.3±0.5	7.2±2.6	2.3±0.5
	M	15737.7±9300.3	1209.8±606.4	6870.4±1786.5	637.6±323.1	24.9±14.6	2.7±0.8	38.7±8.1	21.1±4.1
AUC_{inf} (h*ng/ml)	B	8129.0±906.2	486.8±96.9	3615.4±872.3	239.9±82.1	17.0±2.0	1.8±0.6	7.4±2.6	2.4±0.5
	M	16842.2±9089.7	2157.4±785.0	8380.9±1944.5	695.7±362.8	29.3±13.8	3.4±0.7	39.2±8.0	24.6±6.1
$AUC_{\%Extrap}$ (%)	B	2.9±2.3	10.3±6.8	9.6±6.6	11.5±1.5	6.2±2.4	25.9±15.5	2.7±2.3	5.3±3.4
	M	2.7±0.5	46.2±12.3	17.3±13.8	8.9±2.9	17.6±8.0	10.7±7.0	1.3±0.7	13.4±8.6
V_z/F (L/kg)	B	39.6±7.1	247.8±140.4	118.7±58.0	235.1±94.2	339.5±97.3	145349.8±40480.7	4013.3±1353.8	2496.7±1078.9
	M	58.8±25.2	322.4±22.0	188.6±72.3	372.2±86.7	607.8±396.5	118054.1±38377.6	1706.5±666.9	2822.5±507.0
CL/F (L/h/kg)	B	15.5±1.7	50.7±13.5	26.5±7.0	411.5±160.9	142.6±15.9	18437.4±7250.1	763.9±114.0	1089.6±241.5
	M	8.7±3.2	12.1±4.2	11.4±2.7	225.4±108.6	923.7±274.2	9105.5±2316.3	164.6±28.8	107.4±27.0
$AUMC_{last}$ (h*h*ng/ml)	B	34124.1±4967.9	1935.2±487.3	15357.0±3545.6	192.5±74.8	29.5±5.8	8.9±8.6	31.8±14.3	7.1±2.7
	M	175077.1±103375.1	18194.0±6706.5	92756.1±44786.8	1231.9±677.2	97.9±55.4	22.6±8.6	442.5±232.9	365.4±192.9
MRT_{last} (h)	B	4.3±0.3	4.4±0.5	4.7±0.5	0.9±0.1	1.8±0.1	5.7±3.5	4.3±0.8	3.0±0.6
	M	10.1±2.3	12.5±2.3	12.9±3.0	1.9±0.8	4.1±1.9	8.4±3.1	11.0±3.5	16.5±6.2

B, normal group with HLJDD; M, inflamed group with HLJDD

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density, with a red color in the range of -0.4 to -0.1 (negative value), indicating the inhibitory effect of PK1 on PD1. Furthermore, the relationship between pharmacokinetics and pharmacodynamics was depicted based on the PK-PD analysis. As shown in Fig 6, the relationship between the PK indexes and PD indexes was not simply linearly related, demonstrating a complex interrelationship and the delayed effect of PK indexes. Interestingly, 5 PK indexes of flavonoids, including PK1 (baicalin), PK2 (oroxylin A-7-O-glucuronide), PK3 (wogonoside), PK7
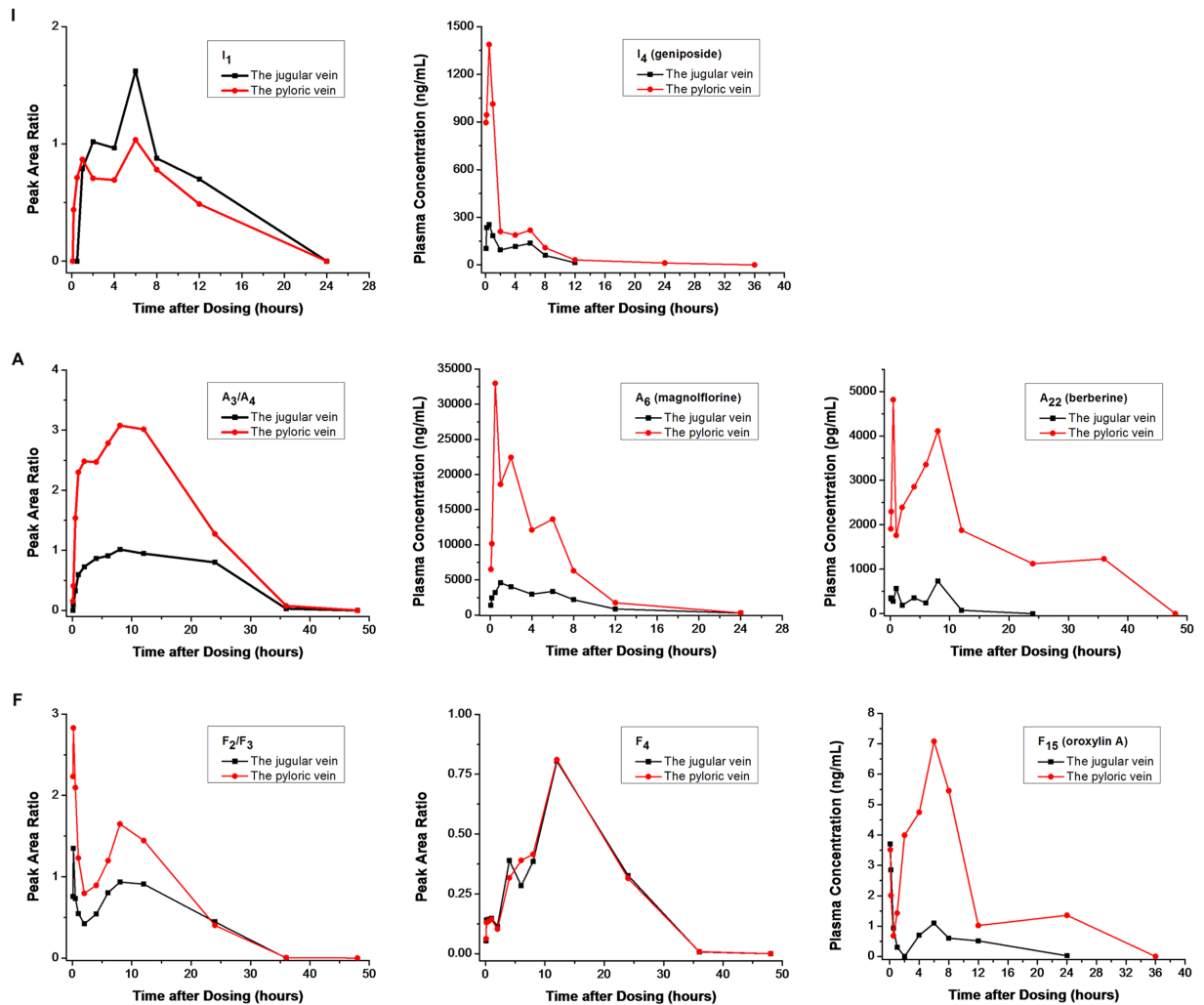
Fig 2. The representative concentration and peak area ratio-time curves of the typical components in the plasma from the jugular vein and the pyloric vein of the inflamed rats (I, iridoids; A, alkaloids; F, flavonoids).

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(wogonin) and PK8 (oroxylin A), presented similar mechanisms of regulating the PD indexes. In comparison, the PK indexes of iridoid (PK4, geniposide) and alkaloids, including PK5 (magnolflorine) and PK6 (berberine), may employ the other mechanism. These results provide a new perspective for elucidating the active substances of HLJDD and their mechanism of treating inflammation.

Discussion

In the pharmacokinetic study, the flavonoids demonstrated a bimodal phenomenon. The results, especially those for baicalin and wogonoside, were in accordance with those of a previous report [22]. Baicalin was first hydrolyzed into baicalein by β-glucuronidase in the intestinal tract. Then, baicalein was absorbed and metabolized to baicalin in the liver. The baicalin formed in the liver was excreted into the duodenum with bile, which was re-absorbed from the intestine via the enterohepatic circulation [45–47]. Additionally, baicalin was excreted into the gut lumen through multi-drug resistance-associated protein2 (MRP2) after transformation in the intestinal
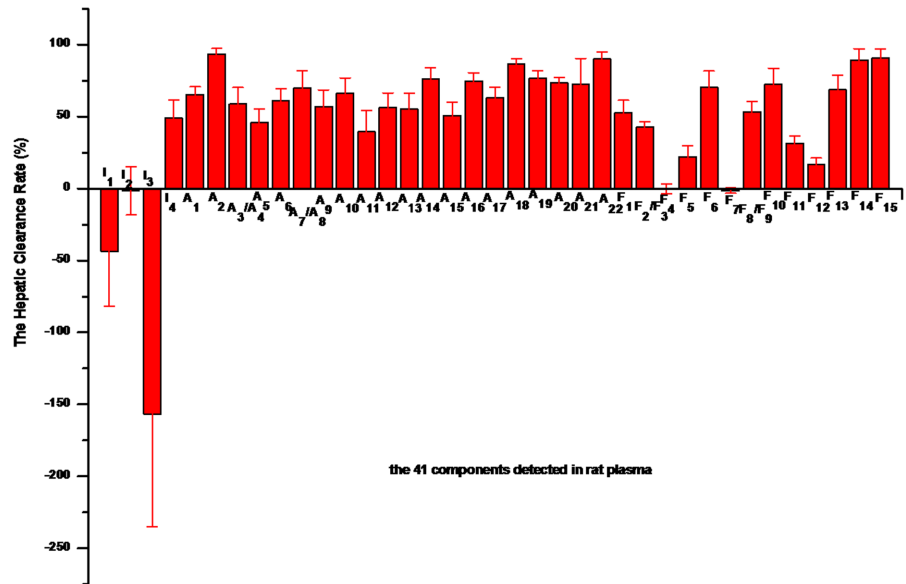
Fig 3. The hepatic clearance rates of the 41 constituents in inflamed rats.

mucosa [48]. Furthermore, protein combination [49] and the absorption of flavonoids at two sites have also been associated with the bimodal phenomenon of flavonoids. For example, it was reported that the first absorption site of baicalin was the upper intestine and the second site was the colon [50]. All of the above findings may have accounted for the double peak phenomenon of flavonoids in rats after the oral administration of HLJDD.

In the present study, the pharmacokinetic parameters of the 41 compounds in inflamed rats were different from those in normal rats after the oral administration of HLJDD. This difference may have resulted from the influence of the pathological status [22, 43, 51], which led to alterations in the function of drug metabolizing enzymes, such as cytochrome P450, β-glucuronidase and UDP-glucuronosyl transferase, together with the expression of drug transporters
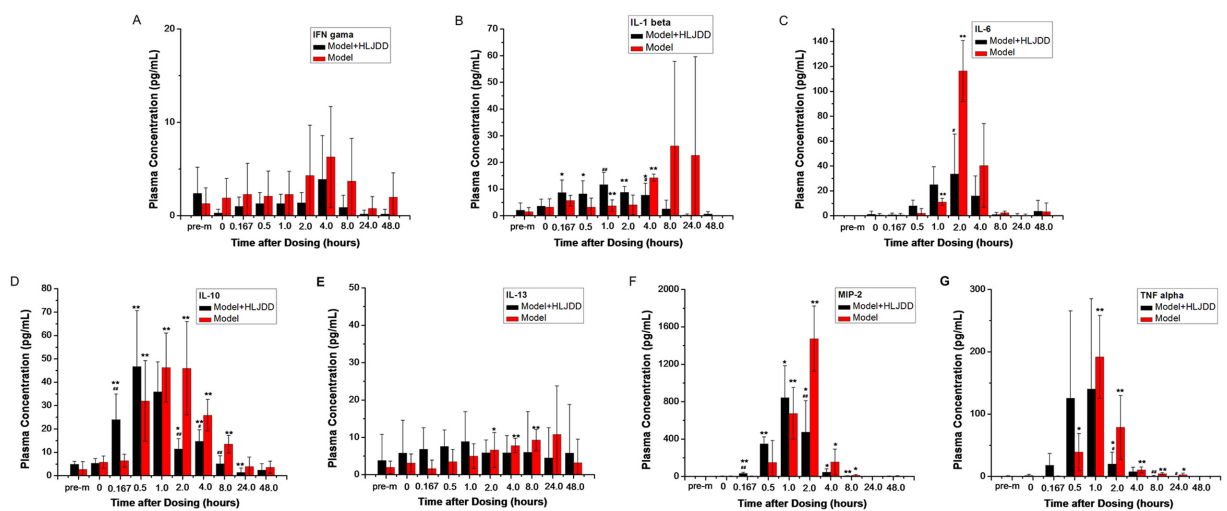
Fig 4. The levels of the 7 cytokines (IL-6, IL-1β, MIP-2, IFN-γ, TNFα, IL-13 and IL-10) in G3 and G4 rats (pre-m, pre-modeling of inflammation; *P<0.05,**P<0.01, before and after modeling; #P<0.05, ##P<0.01, differences between G3 and G4).

Table 2. Measurement of the body temperature of G3 and G4 rats (n = 5).

Time	Body temperature of rats in G3 (°C)	Body temperature of rats in G4 (°C)
Pre-modeling of inflammation	36.45±0.45	36.14±0.29
After modeling of inflammation		
0 min	36.83±0.09	36.68±0.49
10 min	36.84±0.19	36.39±0.73
30 min	36.50±0.18	36.36±0.58
1 h	36.49±0.12	36.53±0.21*
2 h	36.48±0.25	36.41±0.14
4 h	37.60±0.30**	37.77±0.58**
8 h	37.68±0.19**	36.87±0.51* ##
24 h	35.87±0.50	36.08±0.06
48 h	36.07±0.33	35.98±0.11

*$P<0.05$,

**$P<0.01$ (before and after modeling);

#$P<0.05$,

##$P<0.01$ (differences between G3 and G4)

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like P-glycoprotein [52, 53]. In addition, the permeability of the vascular and intestinal mucosal barrier was enhanced in inflamed rats. Thus, the absorption of drugs increased and elimination became slower.

Based on the difference in the blood concentrations between the input and output of the liver, the local hepatic metabolism of HLJDD was evaluated by HCR experiments. According to the results, all of the alkaloids and most of the flavonoids exhibited a relatively higher HCR than iridoids. The HCR values of I_1 and I_3 were -43.7% and -156.6%, respectively, while that of I_2 was close to zero. Many oral drugs are metabolized in the digestive tract and liver before they travel to the target organs [54]. The low bioavailability of oral drugs is due to intestinal
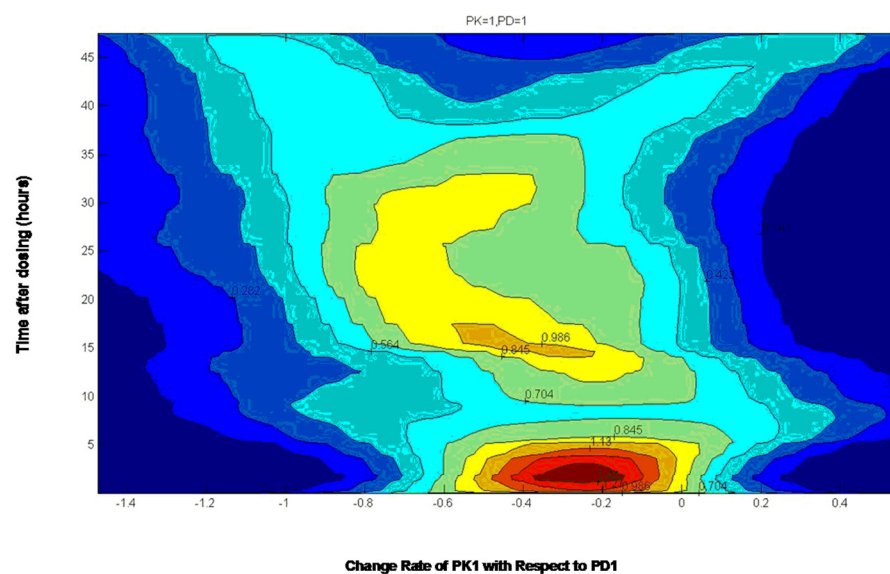

Fig 5. The average change rate of PK1 (baicalin) with respect to PD1 (IL-6). The brightness of the colors represents the probability density of the related indexes.
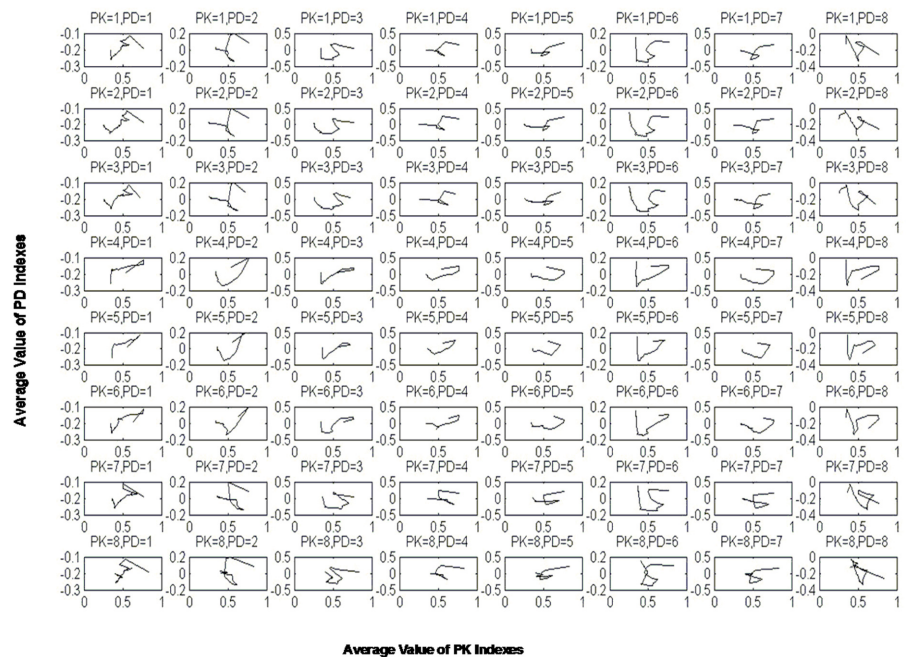
doi:10.1371/journal.pone.0156256.g005

Fig 6. The relationship between pharmacokinetics and pharmacodynamics. Each subgraphic represents the change in the trend of a PD index along with a PK index; PK indexes 1–8 represent baicalin, oroxylin A-7-O-glucuronide, wogonoside, geniposide, magnolflorine, berberine, wogonin and oroxylin A, respectively; PD indexes 1–8 represent IL-6, IFN-γ, IL-1β, MIP-2, TNF-α, IL-13, IL-10 and the body temperature of rats, respectively.

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bioconversion [55] or the liver and intestinal first-pass effect [40, 56–58]. The HCR and pharmacokinetic behaviors of the main ingredients in HLJDD provide beneficial guidance for its clinical use.

HLJDD was first recorded in the book "Wai Tai Mi Yao" by Wang Tao in the Tang Dynasty. Its four herbs have been officially listed in the Chinese Pharmacopoeia. HLJDD is commonly used in the treatment of inflammation-related diseases, such as systemic inflammatory response syndrome [59], acute gouty arthritis [60] and ulcerative colitis [61]. According to pharmacological researches, the anti-inflammatory activity of HLJDD plays a significant role in cecal ligation, puncture-induced liver and lung injury [9], type II diabetes [13, 62] and cardiac damage induced by metabolic disorder in rats [10]. Both the pure compounds [2, 16, 30–34] and the extracts in HLJDD [63]exerted positive effects on inflammation. Three major types of active substances (iridoids, flavonoids and alkaloids) present a synergistic anti-inflammatory effect [16]. However, the relationship between the PK behaviors of various types of constituents absorbed in the plasma and their effects is still not clear. The development of advanced instruments and the time-concentration-effect analysis of HLJDD is necessary. Therefore, our study sought to elucidate the anti-inflammatory effect of HLJDD and aimed to clarify the time-concentration-effect of HLJDD in an animal model of inflammation. It is generally known that inflammatory CKs can be used for the evaluation of inflammation and prognosis [28, 44, 64]. Meanwhile, HLJDD may block the release of inflammatory mediators [65] and reduce the levels of the inflammation-related cytokines TNF-α, IFN-γ, IL-1β, IL-6 and IL-17 in the inflammation model [6, 9, 20, 66]. HLJDD may inhibit Th17 activation, reverse the shift from the Th1 to Th2 response, promote the Th1/Th2 balance toward Th1 predominance [9] and inhibit the activation of NF-κB [10, 30]. Our research demonstrated that HLJDD may significantly

reduce the expression of MIP-2, TNF-α and IL-6 at 2 h, IL-1β at 4 h and IL-10 at 2–8 h in inflamed rats and down-regulate the body temperature at 8 h to achieve its anti-inflammatory effect. In logistic transition mathematical analysis, the flavonoids displayed a similar mechanism of action, while iridoids and alkaloids employed another mechanism. These results supported the consensus that the pharmacological activities of the same type of compounds were similar to each other [67]. There is a growing consensus that TCM is a complex system, and a single component is not sufficient to characterize its pharmacokinetic process *in vivo*. The active prototypes of TCM and their metabolites *in vivo* constitute the material foundation of medicinal effectiveness. Based on our previous studies [37, 38], a total of 41 constituents, including flavonoids, alkaloids and iridoids, were detected in rat plasma. Among the 41 compounds, some were prototypes and the others were metabolites. Thus, they were chosen as pharmacokinetic markers to comprehensively illustrate the pharmacokinetic behavior of HLJDD *in vivo* and subsequently investigate the correlation of different types of compounds (flavonoids, alkaloids, iridoids, prototypes and metabolites) between the exposure dose and their anti-inflammatory effects in rats. The present study provided meaningful evidence to facilitate the understanding of the anti-inflammatory activity of HLJDD as an integrated system.

Conclusions

In summary, the pharmacokinetic behavior and HCR of the main ingredients in HLJDD were investigated in this paper. A time-concentration-effect PK-PD analysis of HLJDD in an acute inflammation model was developed. The pharmacokinetic parameters (especially AUCs and C_{max}) in inflamed rats were significantly increased compared to those in normal rats. The HCR of the major components of HLJDD were approximately 50%, demonstrating a moderate liver first-pass effect on HLJDD *in vivo*. This provides beneficial guidance for its clinical use. The mechanism by which HLJDD synergistically treats inflammation with its three major types of compounds (alkaloids, flavonoids and iridoids) was successfully verified. In addition, a visual PK-PD mathematical model was established to verify the accuracy of the results. This study provided corroborative evidence and a comprehensive understanding of the anti-inflammatory effects of HLJDD for clinical applications. Research on TCM formulas and the relationship between responses and exposure doses will facilitate the development of future clinical applications of these compounds.

Supporting Information

S1 Fig. The extracted chromatograms of 41 constituents in samples. Samples from left to right are rat plasma after oral administration of HLJDD, extract of HLJDD and blank plasma of rats.
(PDF)

S2 Fig. The peak area ratio-time curves of the other 33 ingredients in normal and inflammation rats.
(PDF)

S1 Table. The selected detecting ions, collision energy (CE) and detecting conditions of the analytes.
(DOCX)

S2 Table. Calibration curves and LODs of geniposide, magnolflorine, baicalin, berberine, oroxylin A-7-O-glucuronide, wogonoside, wogonin and oroxylin A.
(DOCX)

S3 Table. Precision and accuracy of geniposide, magnolflorine, baicalin, berberine, oroxylin A-7-O-glucuronide, wogonoside, wogonin and oroxylin A.
(DOCX)

S4 Table. Stability of geniposide, magnolflorine, baicalin, berberine, oroxylin A-7-O-glucuronide, wogonoside, wogonin and oroxylin A in rat plasma (n = 3).
(DOCX)

S5 Table. Extraction recovery and matrix effect of geniposide, magnolflorine, baicalin, berberine, oroxylin A-7-O-glucuronide, wogonoside, wogonin and oroxylin A in rat plasma (n = 6).
(DOCX)

Author Contributions

Conceived and designed the experiments: HYZ YJH BLB. Performed the experiments: WR RZ. Analyzed the data: RZ SKX LYH YYZ WR. Contributed reagents/materials/analysis tools: HJW NS JY YNW SYH BG HH. Wrote the paper: RZ SKX LYH YYZ WR.

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