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Determination and pharmacokinetics of isoferulic acid in rat plasma by high-performance liquid chromatography after oral administration of isoferulic acid and *Rhizoma Cimicifugae* extract

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Abstract

A simple and specific high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) absorbance detection has been developed for the determination of isoferulic acid in rat plasma. The plasma samples were deproteinized with methanol after the addition of internal standard (IS) tinidazole. The analysis was performed on a Kromasil C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size) with acetonitrile–0.05% phosphoric acid (25:75, v/v) as mobile phase. The linear range was 0.0206–5.15 μg ml^{-1} and the lower limit of quantification (LLOQ) was 0.0206 μg ml^{-1}. The intra- and inter-day relative standard deviations (R.S.D.s%) were less than 11.4 and 12.3%, respectively, and accuracy as relative error (R.E.%) between −6.7 and −1.1%. Mean extraction recovery was above 80%. The validated method was successfully applied to the pharmacokinetic study of isoferulic acid in rat plasma after oral administration of isoferulic acid and *Rhizoma Cimicifugae* extract.

Keywords: Isoferulic acid; HPLC; Rat plasma; *Rhizoma Cimicifugae* extract

1. Introduction

Rhizoma Cimicifugae (the rhizomes of *Cimicifuga simplex*, *C. dahurica*, *C. heracleifolia*, and *C. foetida*, Ranunculaceae) is used as antipyretic, analgesic, and wound healing agent [1] along with other crude drugs in Traditional Chinese Medicine. Isoferulic acid, one of the active components in *Rhizoma Cimicifugae*, has been reported to have an antihyperglycemic action on spontaneously diabetic BioBreeding/Worcester (BB/W) rats [2], a plasma glucose-lowering effect in streptozotocin-induced diabetic rats (STZ-diabetic rats) [3], an improving effect on glucose use in skeletal muscle and a reduction of hepatic gluconeogenesis in rats with an insulin deficiency [2]. These observations suggest that isoferulic acid could be effective for the treatment of diabetes [4]. Meanwhile, it has been reported that isoferulic acid improved the survival rate of lethal influenza virus pneumonia in mice [5] and had inhibitory effects on macrophage inflammatory protein-2 (MIP-2) production in response to respiratory syncytial virus (RSV) infection [6]. As to our knowledge, there have been three methods concerning the determination and pharmacokinetics of isoferulic acid [7–9]. One is HPLC method using 12-channel coulometric array detection to determine six metabolites derived from artichoke leaf extract in human plasma, among which isoferulic acid is one metabolite, with the lower limit of quantification (LLOQ) of 0.0011 μg/ml, but the analysis time was about 53 min for isoferulic acid. [7,8]. The other HPLC-UV method established biomarkers of bioavailability and metabolism of hydroxycinnamate derivatives through the identification of the metabolites in human urine and the pharmacokinetics of their urinary elimination, revealing isoferulic acid as a novel marker of caffeoyl quinic acid metabolism and the analysis time of isoferulic acid was also long, about 37 min [9]. In the above methods, isoferulic acid was identified as a metabolite of the phenolic compound and existed in the form of conjugate, which was confirmed by the β-glucuronidase treatment before the sample preparation procedure, protein precipitation and liquid–liquid extraction, respectively [7–9]. In this paper, a direct, specific and rapid HPLC-UV method with a simple protein precipitation procedure and a short run time of about 9 min was described to determine isoferulic acid in rat plasma

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for the first time. The LLOQ of this method is 0.0206 μg/ml. The method was validated and successfully applied to the comparative pharmacokinetic study of isoferulic acid in rat plasma after oral administration of isoferulic acid and *Cimicifuga rhizomes* extract.

2. Experimental

2.1. Chemicals and reagents

Rhizoma Cimicifugae was purchased from Qinghai and identified by Professor Qishi Sun (Department of Pharmacognosy, Shenyang Pharmaceutical University, Shenyang, China). Isoferulic acid was a gift from ACROS ORGANICS (Morris Plains, NJ). Tinidazole (IS) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and phosphoric acid were of HPLC grade; all other reagents were of analytical grade. Redistilled water was used. Blank rat plasma was obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China).

2.2. Animals

Male Wistar rats (200–220 g) were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). They were kept in an environmentally controlled breeding room for 3 days before starting the experiments. They were fed with standard laboratory food and water ad libitum and fasted overnight before the test. All procedures involving animals were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China.

2.3. Chromatographic system

HPLC was performed with a Shimadzu LC-10ATVP series instrument (Tokyo, Japan) composed of a double quaternary gradient system, column oven, SPD-M10AVP detector, and Class-VP was used for data collection. Chromatographic separation was performed on a Kromasil C_{18} column (250 mm × 4.6 mm i.d., 5 μm particles, Dalian, China). The mobile phase was acetonitrile–0.05% phosphoric acid (25:75, v/v). The analysis was carried out at a flow rate of 1.0 ml min^{-1} with detector wavelength set at 320 nm. The column temperature was maintained at 35 °C.

2.4. Preparation of calibration standard and quality control samples

Stock solution of isoferulic acid was prepared in methanol at the concentration of 206 μg/ml, stored at 4 °C and was further diluted in methanol to make working standards. Stock solution of internal standard was prepared in methanol and diluted at the concentration of 4.2 μg/ml with methanol to prepare the working solution containing 4.2 μg/ml of tinidazole. Calibration samples were prepared by addition of working standards of isoferulic acid to blank plasma giving final concentrations of 0.0206, 0.0515, 0.103, 0.515, 2.06 and 5.15 μg/ml. The quality control (QC) samples were prepared from separate stock solutions in blank plasma at the concentrations of 0.0515, 0.515 and 4.12 μg/ml. The spiked plasma samples were stored at −20 °C prior to analysis.

2.5. Preparation of plasma samples

To 200 μl of plasma, 50 μl of internal standard solution (tinidazole, 4.2 μg/ml) and 350 μl of methanol was added and vortexed for 5 min. After centrifugation at 12,000 × *g* for 10 min, the supernatant was transferred to a clean test tube and evaporated to dryness in a water bath at 40 °C under a stream of nitrogen. The residue was reconstituted in 100 μl methanol, with vortex mixing for 60 s, and the centrifugation procedure was then repeated. An aliquot of 20 μl of sample of the supernatant was injected into the HPLC system for analysis.

2.6. Method validation

2.6.1. Selectivity

Selectivity was investigated by comparing chromatograms of blank plasma obtained from six rats with those of corresponding standard plasma sample spiked with isoferulic acid and IS and plasma sample after an oral dose. All six blank plasma samples were extracted to ensure the absence of interfering peaks.

2.6.2. Linearity and LLOQ

To evaluate linearity, calibration standards in plasma at six concentration levels of isoferulic acid ranged 0.0206–5.15 μg/ml were prepared and assayed on three consecutive days. Calibration curves for isoferulic acid in plasma were generated by plotting the peak area ratios (y) of isoferulic acid to tinidazole versus those nominal concentrations (x) in standard plasma by the $1/x^2$ weighted least-square linear regression. The lowest standard on the calibration curve should be accepted as LLOQ if the following two conditions are met: one is that the analyte response at the LLOQ should be at least 5 times the response compared to blank response, the other is that analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120% [10].

2.6.3. Precision and accuracy

The precision and accuracy were assessed by determining quality control (QC) samples at three concentration levels of isoferulic acid (0.0515, 0.515 and 4.12 μg/ml) on three consecutive days. Precision was expressed as relative standard deviation (R.S.D.%) and accuracy as relative error (R.E.%). Intra-day precision and accuracy were determined by repeated analysis of a set of QC samples on one day ($n = 6$), while inter-day precision and accuracy by repeated analysis on three consecutive days ($n = 6$, series per day), using standard curve prepared required to be within 15%.

2.6.4. Extraction recovery

Recovery of isoferulic acid was evaluated at three concentrations levels of QC samples (0.0515, 0.515 and 4.12 μg/ml) and for IS (4.2 μg/ml) on the same day. Recovery data were determined by comparing the peak area ($n = 6$ at each concentration) obtained from plasma samples spiked with analyte before extraction with those from the corresponding standards diluted with water.

2.6.5. Stability

The stability of isoferulic acid and IS stock solutions was evaluated after storage at room temperature and at 4 °C for 30 days. The stability of isoferulic acid and IS working solutions was investigated at room temperature for 5 h. QC plasma samples of three concentration levels were (0.0515, 0.515 and 4.12 μg/ml) subjected to the conditions below. Short-term stability was assessed by analyzing QC plasma samples kept at room temperature for 4 h that exceeded the routine preparation time of samples. Long-term stability was determined by assaying QC plasma samples after storage at −20 °C for 14 days. Freeze–thaw stability was investigated after three freeze (−20 °C)–thaw (room temperature) cycles. Post-preparative stability was assessed by analyzing the extracted QC plasma samples kept at room temperature for 4 h.

2.7. Application of the HPLC-UV method and pharmacokinetic study

The method was used to determine isoferulic acid in rat plasma after oral administration of isoferulic acid and *Rhizoma Cimicifugae* extract at a dose of 3 mg/kg. Animals were divided into two groups. Each group included six male rats. Blood samples (0.5 ml) were collected from the abdominal vein before dosing (0 min) and 1, 3, 5, 7, 9, 12, 16, 20, 30, 45, 60, 90, 120, 180 and 240 min after dosing then immediately transferred to heparinized tubes and centrifuged at 3000 × *g* for 10 min. The plasma obtained was stored at −20 °C until analysis. The plasma concentrations of isoferulic acid at different times were expressed as mean ± S.D. and the mean concentration–time curve was plotted. All data were processed by noncompartmental analysis using the DAS 2.0 software package (Chinese Pharmacological Society). The maximum plasma concentration (C_{max}) and the time to reach this concentration (t_{max}) were obtained directly from the observed values. The apparent elimination rate constant (K_e) was calculated by fitting mean data at four terminal points of the plasma concentration profile with a log–linear regression equation using the least-squares method. The plasma half-life, $t_{1/2}$, was calculated as $0.693/K_e$. The area under the plasma concentration–time curve from zero to the time of the final measurable sample ($AUC_{0–t}$) was calculated using the log linear-trapezoidal rule up to the last sampling point with a detectable level (C). The area under the plasma concentration–time curve from zero to infinity ($AUC_{0–\infty}$) was calculated using the log linear-trapezoidal with extrapolation to infinity.

3. Results and discussion

3.1. Method development

The mobile phase composed of 0.05% acetonitrile–phosphoric acid (25:75, v/v) was found to be appropriate for the analysis of isoferulic acid. The retention time for isoferulic acid and IS was 6.1 ± 0.04 and 8.5 ± 0.05 min, respectively ($n = 6$). The appropriate composition of phosphoric acid was necessary to achieve satisfactory peak symmetry and resolution. Eventually, the 0.05% phosphoric acid and the final composition of the mobile phase was chosen to achieve the acceptable peak shape, appropriate retention time and satisfactory separation of isoferulic acid from endogenous components in rat plasma.

The specificity of the method for the determination of isoferulic acid was investigated by peak purity check using the diode array UV detector. No interference from *Rhizoma Cimicifugae* was observed at the retention times of the analytes.

Liquid–liquid extraction and protein precipitation are the commonly used extraction techniques. Liquid–liquid extraction usually offers much cleaner sample that in turn makes the method more robust and scalable. However, in the process of our method development, several extraction solvents were tested, including ethyl ether, ethyl acetate, and chloroform. We found that the use of liquid–liquid extraction technique resulted in a relative low recovery for isoferulic acid and IS which may be attributed to their relatively high polarity. Then the protein precipitation method was considered. A variety of plasma protein precipitating reagent, e.g. methanol, ethanol, acetonitrile and 10% trichloroacetic acid were evaluated. Methanol was found to be optimum, which provided a clean supernatant with acceptable extraction recovery for isoferulic acid and internal standard without interference peaks. The methods published by Wittemer et al. involved a sample precipitation of 500 μl of human plasma with 600 μl of acetonitrile and got the mean recovery of about 71% for isoferulic acid [7,8]. Our method used 200 μl of rat plasma and got the mean recovery of about 81.9% for isoferulic acid.

3.2. Method validation

3.2.1. Selectivity

The specificity of the method was determined by comparing the chromatograms of blank plasma with the corresponding spiked plasma. Typical chromatograms of a blank plasma sample, a spiked plasma standard and a plasma sample from a rat 7 min after dose are shown in Fig. 1. No interferences from endogenous substances were observed at the retention time regions of the analyte and IS. The retention times of isoferulic acid and IS were 6.1 and 8.5 min, respectively.

3.2.2. Linearity and LLOQ

Linear calibration curves were obtained over the concentration range 0.0206–5.15 μg/ml for isoferulic acid in rat plasma. A typical calibration plot equation was $y = 0.002143x + 0.01626$ with a correlation coefficient of 0.9965. The lower limit of quantification (LLOQ) for isoferulic acid in rat plasma was

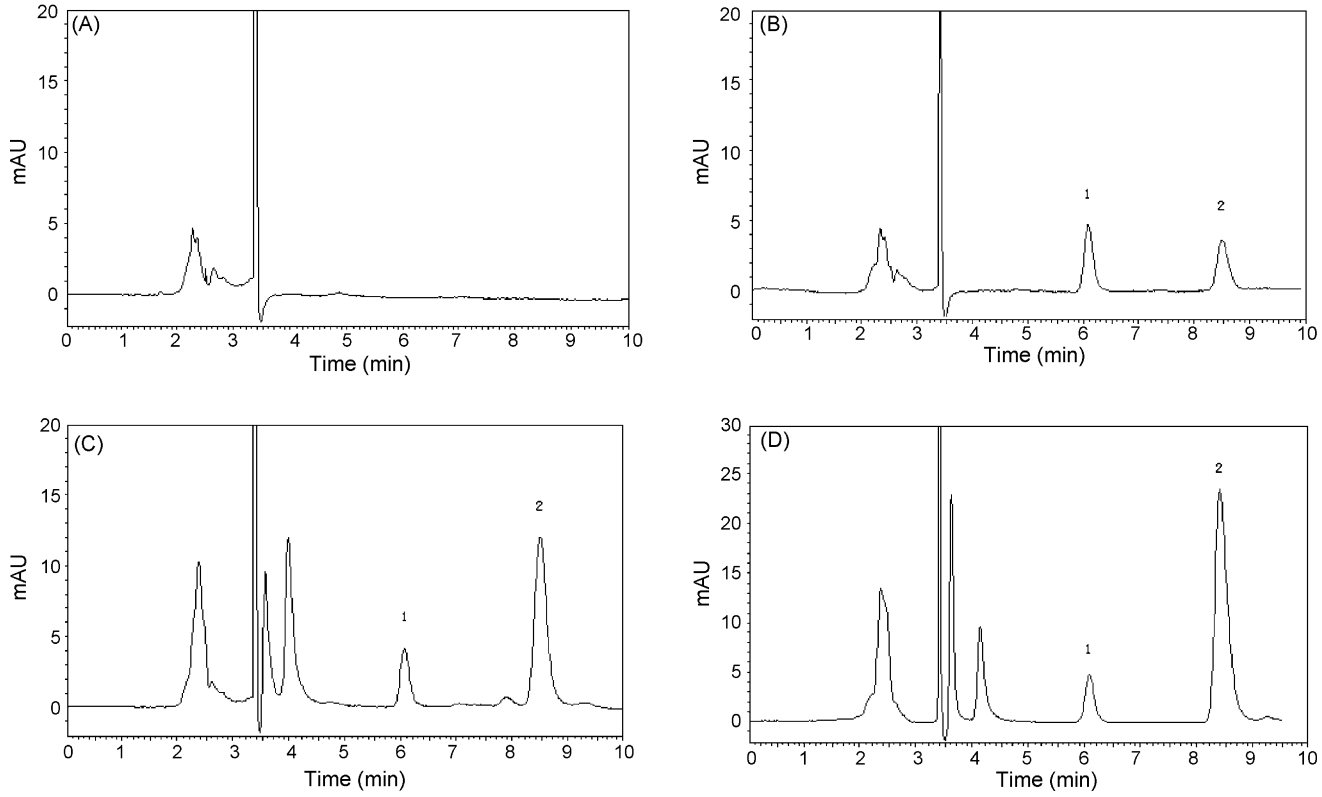

Fig. 1. Chromatograms of (A) a blank plasma sample, (B) a blank plasma sample spiked with isoferulic acid (0.515 μg/ml) and internal standard (1.05 μg/ml), (C) a plasma sample from a rat 7 min after oral administration of isoferulic acid and (D) a plasma sample from a rat 7 min after oral administration of *Rhizoma Cimicifugae* extract. Peak 1, tinidazole (IS); peak 2, isoferulic acid.

0.0206 μg/ml with precision and accuracy presented in Table 1. Results of R.S.D. (%) and R.E. (%) for HPLC determination of isoferulic acid in rat plasma during method validation were presented in Table 2.

Table 1
Precision and accuracy for the determination of isoferulic acid in rat plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days)

Nominal C (μg/ml)	Found C (μg/ml)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Accuracy R.E. (%)
0.0515	0.0495 ± 0.0033	2.3	7.1	3.9
0.515	0.481 ± 0.026	11.4	3.9	6.7
4.12	4.07 ± 0.24	2.4	6.3	1.1

Table 2
Results of R.S.D. (%) and R.E. (%) for HPLC determination of isoferulic acid in rat plasma

	Nominal C (μg/ml)					
	0.0206	0.0515	0.103	0.515	2.06	5.15
Back-calculated concentration	0.0202	0.0542	0.104	0.492	2.01	5.30
	0.0208	0.0491	0.109	0.514	1.94	5.36
	0.0209	0.0497	0.101	0.533	1.90	5.55
Mean	0.0206	0.0510	0.105	0.513	1.95	5.40
R.S.D. (%)	2.0	5.5	3.6	4.0	2.8	2.4
R.E. (%)	0.1	−1.0	1.6	−0.4	−5.3	4.9

The methods published by Wittemer et al. had a LLOQ of 1.1 ng/ml for the determination of isoferulic acid in human plasma. The higher sensitivity may not only be attributable to the 500 μl of human plasma which is 2.5-fold the volume of rat plasma we used but also the 12-channel coulometric array detection which was found to be an appropriate detection system for the highly sensitive and selective determination of polyphenols in biological matrix [11–13]. What is more, the multi-channel array detector they used provided the highest selectivity [7].

3.2.3. Precision and accuracy

The intra- and inter-day precision and accuracy results are shown in Table 1. The intra- and inter-day precisions were less than 11.4 and 12.3%, while the accuracy (R.E.) between −6.7 and −1.1%, indicating acceptable precision and accuracy of the method.

3.2.4. Extraction recovery

The results showed that extraction recoveries of isoferulic acid from rat plasma were 80.4 ± 4.5, 82.4 ± 7.4, and 83.0 ± 4.7% at concentrations levels of 0.0515, 0.515 and 4.12 μg/ml, respectively. The mean recovery was 81.9 ± 5.5%. Recovery of the IS was 80.4 ± 4.1% at the concentration used (4.2 μg/ml).

3.2.5. Stability

The stock solutions of isoferulic acid and IS were found to be stable at room temperature for 6 h and at 4 °C for 30 days.

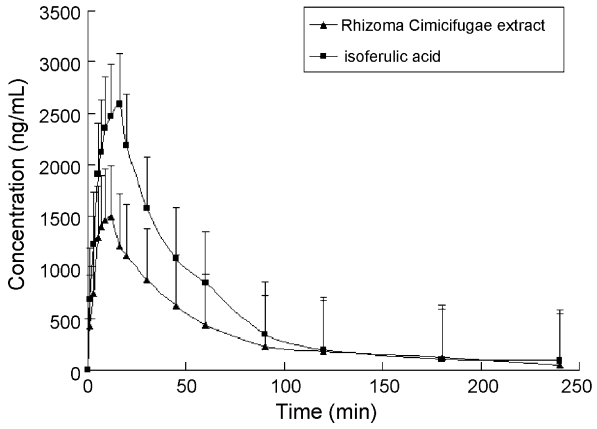

Fig. 2. Mean ± S.D. plasma concentration–time curve for isoferulic acid in rats after oral administration of isoferulic acid and *Rhizoma Cimicifugae* extract at a dose containing 3 mg/kg of isoferulic acid ($n = 6$).

Both working solutions were stable at room temperature for 5 h. Table 2 summarizes the results of short-term stability, long-term stability, freeze–thaw stability of isoferulic acid in plasma and post-preparative stability. All the results well met the criterion for stability measurements.

3.3. Pharmacokinetic application

The developed method has been successfully used for the pharmacokinetic study of isoferulic acid in rat plasma after oral administration of isoferulic acid and *Rhizoma Cimicifugae* extract (the content of isoferulic acid is 2 mg/g). The concentration–time curves (mean ± S.D.) of isoferulic acid after oral administration of isoferulic acid and *Rhizoma Cimicifugae* extract in rats are shown in Fig. 2 and the corresponding pharmacokinetic data are shown in Table 3. There were significant differences in pharmacokinetic parameters between the two preparations (isoferulic acid and *Rhizoma Cimicifugae* extract) of the same isoferulic acid dosage level. The 1.6-fold ($82.7 \pm 49.8\ \mu g\ ml^{-1}$ min versus $134.1 \pm 70.4\ \mu g\ ml^{-1}$ min) enhancements of AUC_{0-t} were observed from *Rhizoma Cimicifugae* extract compared with isoferulic acid at a dosage of 3 mg/kg isoferulic acid. This was probably caused by the effect of the other constituents in *Rhizoma Cimicifugae*. A variety of compounds have been isolated from *Rhizoma Cimicifugae*, including phenolic components, cimigenol, cycloartane triterpene, and saponins [14,15]. These coexisting constituents may improve absorption of isoferulic acid from the gastrointestinal tract.

Table 3
Stability of isoferulic acid in rat plasma at three QC levels ($n = 3$)

Stability	Accuracy (mean ± S.D.)		
	0.0515 (μg/ml)	0.515 (μg/ml)	4.12 (μg/ml)
Short-term stability	0.0496 ± 0.0034	0.513 ± 0.029	4.46 ± 0.10
Long-term stability	0.0482 ± 0.0051	0.466 ± 0.032	4.20 ± 0.30
Freeze–thaw stability	0.0506 ± 0.0045	0.469 ± 0.027	3.67 ± 0.085
Post-preparative stability	0.0502 ± 0.0030	0.473 ± 0.018	4.34 ± 0.15

Table 4
Pharmacokinetic parameters of isoferulic acid in rats after oral administration of isoferulic acid and *Rhizoma Cimicifugae* extract ($n = 6$)

Characteristic	Estimate (mean ± S.D.)	
	Isoferulic acid	*Rhizoma Cimicifugae* extract
t_{max} (min)	10.3	10.2
C_{max} ($\mu g\ ml^{-1}$)	1.6	2.9
$t_{1/2}$ (min)	85.2	63.4
$AUC_{0-\infty}$ ($\mu g\ min\ ml^{-1}$)	89.1	139.9

Wittemer et al. reported that the peak plasma concentrations of total isoferulic acid were reached within 1 h and declined over 24 h showing almost biphasic profiles [8], while in our paper the t_{max} of isoferulic acid was about 10 min, the difference may be attributable to the different kinds of subject, different drugs administrated, and it usually takes time for the parent drug to be metabolized to isoferulic acid in the situation as described in the above paper.

As shown on Fig. 1(C) and (D), two peaks of unknown materials with retention times of 4.1 and 3.6 min appear in the chromatograms while they are not observed in Fig. 1(A) and (B). It suggests that they are two polar substances compared with the parent drug and might be metabolites. If they are glucuronides, the β-glucuronidase treatment may help to confirm the metabolism pathway. But so far we have not been able to identify what they actually are. The metabolism of isoferulic acid in rats needs further study (Table 4).

4. Conclusion

A simple and specific HPLC method with UV detection has been developed for quantitative determination of isoferulic acid in rat plasma. The method was validated for linearity, selectivity, accuracy, precision, LLOQ and recovery. The method was successfully applied to the determination of isoferulic acid in rat plasma after oral administration of isoferulic acid and *Rhizoma Cimicifugae* extract. Significant increase in $AUC_{0-\infty}$ was found by comparing the results after oral administration of *Rhizoma Cimicifugae* extract with that of isoferulic acid at the same dosage level in the comparative pharmacokinetic study. The mechanism is still ambiguous and needs further profound research. The pharmacokinetic results are useful for the further study of the clinical applications of isoferulic acid and *Rhizoma Cimicifugae* extract.

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